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EDITORIAL

In the past year we were faced with many challenges both globally as well as in the editorial office of the *Slovak Journal of Animal Science*, e.g. remote working, numerous restrictions in workspaces as well as in the everyday life and the necessity to safeguard the health of ourselves and our communities.
Despite these restrictions we managed to publish all four full issues of the journal in 2020.

Production of livestock and quality animal products is an essential aspect to maintain the access to healthy and safe food and to ensure food security. Last year has highlighted the fragility of our existing food chains. *Slovak Journal of Animal Science* strives to contribute to the best science and practice by publishing high quality articles on topics across animal science and production.

To best serve this goal and the scientific community, we always strive to improve the quality of our publication process. Over the past few years, since I took over as the Editor-in-Chief, we have been working on improvements to the journal's accessibility and infrastructure to improve the experience for authors, reviewers and readers. The journal now resides on its own website. The Aims and Scope have been updated to best reflect the journal's mission, and the access to all relevant up-to-date information regarding the journal and its publication process has been improved. The manuscript submission and review process has been modernised by implementing Open Journal System.

As a result, SJAS has experienced growth in the number of manuscripts submitted to our journal as well as readers interested in our publication. In order to extent the scope of our journal and to enrich the spectrum of submitted manuscripts, SJAS has decided to expand its Editorial Board. I would like to express my gratitude to the standing Editorial Board members for their relentless work and dedication, and I would like to extend a warm welcome to the new ones. I look forward to working with you.

We want to continue to serve the scientific community with commitment to the research integrity and the highest publishing ethics. Our next steps will lead to the improvement of the journal's accessibility through international databases. There are still challenges ahead of us, but as our progress over the past years has shown, we are ready to meet them. We invite you to submit your work to SJAS.

prof. Ing. Peter Chrenek, DrSc.
Editor-in-Chief

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INNOVATED SURGERY PROTOCOL FOR RUMEN CANNULATION IN RUMINANTS

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ABSTRACT

The manuscript describes surgical technique of rumen cannulation in cattle using flexible cannulas, which are necessary in prediction of experimental feeds' potential degradability using an *in situ* method. The main principles of pre-operative preparation of the experimental animals and their post-operative treatment to ensure their long-term use in experiments with no complications are also discussed. In addition, we provide a brief overview of the history of surgical techniques and procedures used to determine feed digestibility. This overview proves that, despite the efforts to avoid surgeries on experimental animals, these remain actual and necessary.

Key words: ruminants; surgery; rumen cannula; pre- and post-operative treatment; *in situ*

INTRODUCTION

Cannulated ruminants are irreplaceable in evaluation of potential feed degradation, which is determined by the *in situ* method, and in combination with duodenal intestinal cannula it creates the basis to determine the intestinal-enzymatic or total digestibility of feed (Chrenková *et al.*, 2012; 2018). In the past, firm PVC cannulas were used for rumen cannulation, which were composed of several components and were completed during the surgery itself. At present, compact flexible cannulas (patent Bar Diamond Inc., USA) are used. These differ in their diameters, the length of the tube, and diameters of the fixation bases depending on the size of the animal. The flexible cannula requires some changes in the already described cannulating process (Szakács *et al.*, 1990a; b). We consider it important to name the medications available and used at present rather than those, which are no longer manufactured. We updated also the older surgical techniques used in rumen cannulation.

MATERIAL AND METHODS

Rumen cannula

For the surgery, a rumen cannula for small ruminants produced in Bar Diamond, Inc. from the USA (Figure 1) was used. Its description and composition are at the website https://shop.bardiamond.com/en/small-rumen-cannulae (# 8C). Later, it was replaced by a cannula for large ruminants from the same producer https://shop.bardiamond.com/en/large-rumen-cannulae (# 1C).

Preparation of animals

Animals were selected basing on the aim of the research, with the goal of their long-term

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 # 8C **# 1C**

Figure 1. Rumen cannula

utilization. Most suitable are young heifers (up to 250 kg), which have low amounts of subcutaneous and deposit fat, making the surgery much easier. At the first phase, it is necessary to acclimate the animals to tied-up housing as well as daily physical contact in the region of *Fossa paralumbalis*. This daily "training" combined with feeding, cleaning and washing of the animals continues after the surgery as well, with a pause to allow healing of the surgical wound. The experimental animals then do not develop negative association in relation to the short-term pain after the surgery, therefore, later it is not necessary to sedate or fixate them when manipulating the cannula during the experiments. A pre-surgical diet (hay for 48 hours) is necessary when cannulating an animal. Hay and water cease to be provided only in the morning of the surgery. Filled rumen after the evening feeding maintains an almost physiological position of this organ, which makes the surgery considerably easier.

The surgery itself consists of several stages:

Preparation of the surgical site (after shaving of the region of *Fossa paralumbalis* the previous day) consists of mechanical cleaning, degreasing of the surgical field (Benzinalcohol) and then disinfection using Ajatin tincture. The night before the surgery, the ruminal cannula is placed into clean water with Ajatin solution.

Sedation and anaesthesia. Sedation using xylazin (Rometar 2 % inj.) in the amount 0.10 – 0.15 ml.100 kg^{-1} of live weight. Xylazin is characterised, in addition to its analgesic and myorelaxant properties, also by its effect on the motor functions of the digestive tract, therefore, we recommend a minimum dose necessary to calm down and immobilize the animal. High doses may cause not only the animal to lie down but a risk of long-term atony of the foreguts. Therefore, we ensure the quality of the local anaesthesia using 2 % solution of procaine (Procain Bioveta 100 mg.100 ml^{-1} inj., with the effective substance Procaini hydrochloricum 100 mg) at the maximum amount of 60 ml, starting with the subcutaneous layer, through all muscle layers and around the perimeter of the presumed circular incision. After uncovering the peritoneum, a few drops may be applied on its surface as well. Sufficient local anaesthesia is reached in 15 minutes. Before initiating the surgery, the antibiotic Norostrep inj. or Shotapen inj. at recommended doses is applied.

The circular resection of the stomach cavity is always performed after placing a sterilised tube of the cannula, coated with pyotanicin (crystal violet, methylrosanilinii chloridum) to the centre of the left *Fossa paralumbalis*. The incision into skin (cutis) is led

Figure 2. Incision through the skin

along the inner perimeter of the marking (Figure 2). Further, to proceed in the following order: subcutis, tunica flava abdominis and muscle layers (m. cutaenus trunci, m. obliquees externus et internus abdominis, m. transversus abdominis). Veins are ligated with each layer to prevent bleeding into the stomach cavity after peritoneum is cut. After peritoneum is pulled out using a haemostat, we cut through it under hand control using blunt scissors. The muscle and peritoneum should be preserved as much as possible. Using this way, later enlargement of the created stoma by the cannula's weight is prevented.

The uncovered rumen is fixed using haemostat in the wound in the way to avoid larger branches v. ruminalis sinister and prevent damage to branching of vegetative nerves. It is also important to preserve the topography and avoid the entry of the inner disc of the cannula outside the dorsal ruminal sac. If the rumen is almost full, it is lifted directly into the surgery wound and the necessity of fixation is eliminated.

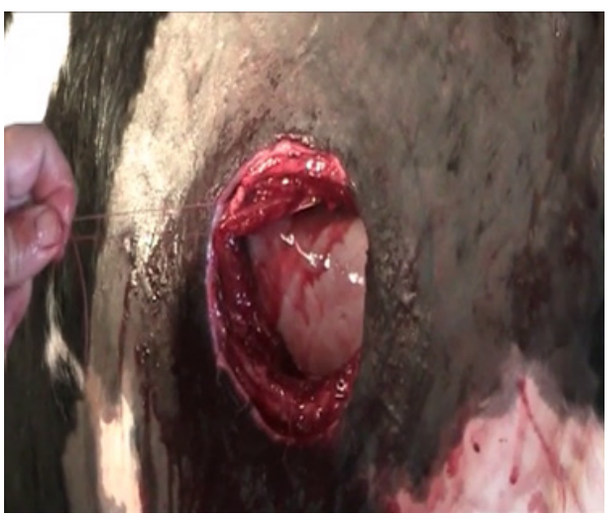

Figure 3. Suturing the rumen wall with the abdominal wall

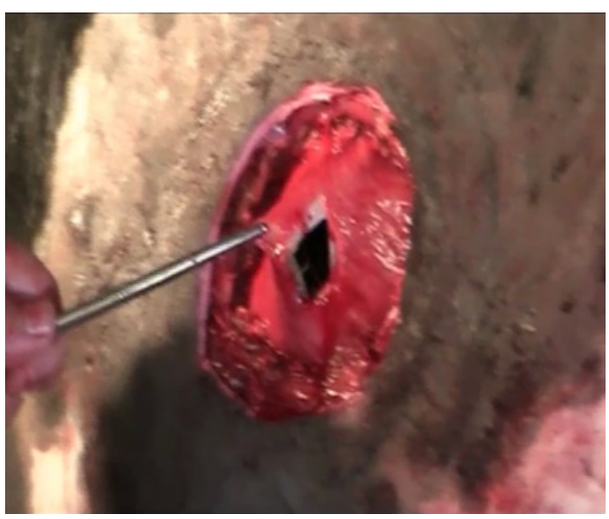

Figure 4. Opening of the rumen after pressurization of the abdominal wall

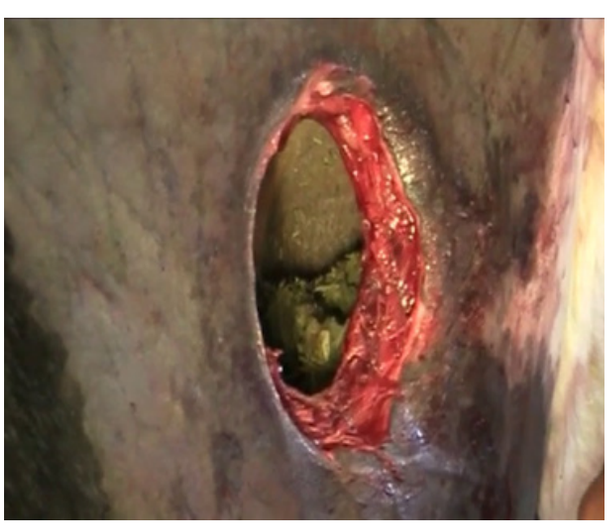

Figure 5. Rumen prepared for insertion of the cannula

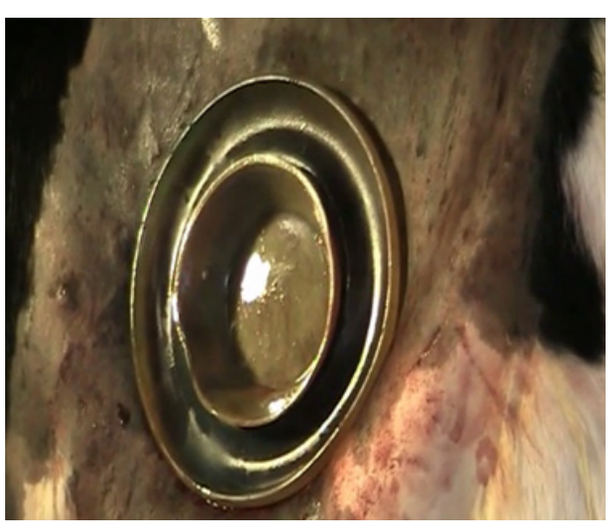

Figure 6. Completed rumen cannula, corked

After hermitization of the stomach cavity, the next phase is dressing of the rumen wall approximately 3 – 4 mm above the sutures, which allows sufficient space for later ligation of veins (Figure 4). This is the phase with the strongest bleeding, even if a site without visible larger veins was selected. The bleeding veins need to be carefully ligated, since the flexible cannula does not provide pressure compression as was the case with tightened disks of a firm cannula.

After treating the dried and cleaned wound with antibiotic ointment (Figure 5), the cannula itself is inserted by deforming the inner fixation disk. The position of the inner disk and its re-straightening in the dorsal ruminal sac is evaluated manually through the tube of the cannula and the tube is corked (Figure 6).

After the surgery, a dose of analgesics (Novasul inj., Richterpharma AG) is administered to prevent the cannula being ripped out by defensive reactions after the local anaesthetics fade. The analgesics are administered for several days after the surgery, as needed, which lowers the post-operative stress and, therefore, speeds up the healing of wounds as well as prevents defensive reactions of the cannulated animals during handling.

During a long-term use of the experimental animals, vitamin injections (ADE-Vit a.u.v. inj., Bioveta, Ivanovice na Hané) are administered regularly; for prevention of liver damage, Menbuton "WERFT" (Sanochemia Pharmaceutika AG) is applied.

Post-operative care

For 2 days after the surgery, quality hay is provided until the atony of rumen, caused by Xylazin, passes entirely. Afterwards, acclimatisation to experimental diet begins (minimum of 14 days). Antibiotics (Norostrep inj.) are administered for 4 – 5 days after the surgery, resp. on the third day after two administrations of Norostrep Shotapen inj. is depot delivered. In case of oedema, it is treated using resorption ointment (Aphlegmin ung.). Skin sutures are not removed; they usually loosen during the healing period through maceration of stoma by the tube when the animals move and by re-established contractions of the rumen. Between the 8th and 10th day, the cannula is removed from the rumen and the rumistoma is examined visually and by palpation. In case of bags, those are treated with antibiotic ointment. Necrotic tissue (remnants of sutures, peritoneum above the sutures) is removed. After cleaning the skin, Chlorophylum spray is applied. The cannula is inserted again and corked. To protect the skin at the site of cannula's entry, dermatologics (Infandolan ung., Indulona) in the form of ointments are applied permanently in order to prevent maceration by leaking rumen fluid. We consider important the daily physical contact of the treating staff in the location of cannula. This "training" completely eliminates later defensive movements and stress of the animal. Using this procedure, the animals are usable for experiments without obstacles for several years.

RESULTS AND DISCUSSION

Technique of rumen cannulation in large and small ruminants has been known for decades (Dougherty, 1965). This surgical technique, with small adjustments, is still used today. Various modifications of surgical procedures and the options to utilize different types of cannulas have been described in detail (Szakács *et al.*, 1990a; b), however those had been always technical aspects and details related to the surgery itself, which were meant to minimalize post-operative complications, secure anaerobic environment in the rumen and, therefore, enable a long-term use of cannulated animals in experiments.

In contrast to Aliev (1974), we did not lead the knots of the sutures of the abdominal wall with rumen through the skin, because we are of the opinion that such sutures covered by the outer disc of the cannula and without air could begin to fester. In case of post-operative oedema, they would also be at risk of tearing. Despite the loss of some portion of muscle mass in the use of a circular incision through the abdominal wall, we rejected also the procedure suggested by Němeček *et al.* (1981). When a horizontal incision through the skin was made, it was necessary to suture it after the surgery, which caused the risk of inflammation of the surgical wound at the lower edge after contact with the secretion from the wound or the rumen fluid containing microflora. In general, attempts to divide the surgery into two stages have also been abandoned, but this option is still being tested (Malik et al., 2015). Size of the rumen cannula and its width depend not only on the type of animal

but its weight as well. We prefer cannulas the tube of which allows access by hand (8 cm), which are replaced with larger ones (12 cm) as the animal grows. Corks are provided with a fixation apparatus to secure bags.

It can be stated that the surgery itself has always been among the simplest in the experimental veterinary surgery practice, but from the aspect of maintaining the function of the operated organ, which can be compared to an anaerobic fermenter, also the most complicated.

Selection of cannula is determined by methodical aims. When rumen fluid needs to be collected for experimental and study purposes, a simple thin cannula is sufficient, but collection of the rumen content requires a tube of a larger size, which would allow an entry by hand. The principle is to minimalize the invasiveness of the surgery on the animal while allowing to achieve goals of experiments.

To clarify, it is necessary to return into the history of development of new testing methods for feed digestibility. *In vivo* methods of balance experiments and marker experiments were intensive in terms of time, labour and costs (Dhanoa *et al.*, 1985). In parallel to searching for cheaper alternatives, the resistance against experiments on animals was also growing. In the 1960s, an *in vitro* method was developed by Tilley and Terry (1963) and it entered mass use, during which it was also being improved upon and factors that could affect the results were also tested (Daniel, 1984; Chrenková, 1984). At the same time, the results obtained by this method were compared to the results gained from *in vivo* experiments (Shqueir *et al.*, 1984). The aim was to develop an artificial rumen (RUSITEC) and the animals would become only donors of rumen fluid with its specific microflora and microfauna (Brice and Morrison, 1984). These authors tried to eliminate the main disadvantage of the *in situ* method: the real processes in the rumen cannot be described using a static model. Rusitec as a dynamic system removed problems with growing mass of bacterial protein, which limited the length of incubation of the experimental material in given environment.

In the 1980s, with the growing interest in temperature and physical methods of treatment to protect plant proteins from their high degradability in rumen, the *in vitro* method showed its limitations. Although it allowed to test the effect of the used treatment on degradability of proteins in rumen, it was impossible to determine, whether these "bypass proteins" affected by Maillard's reaction or higher doses of chemicals in treatments are actually digested by enzymes in the intestines. Developing an *in situ* method to determine rumen degradability of nutrients required a return to the use of cannulated animals (ØRSKOV *et al.*, 1980; Hunter *et al.*, 1981). Animals with a combination of rumen and intestinal cannulas enabled to determine not only the potential degradability of nutrients but their intestinal and total digestibility as well. For this purpose, the mobile bag method was developed (Szakács, 1989). Even in this case, to simplify the method and for the benefit of the animals, the polycannula method (cannulas in duodenum, abomasum and ileum) was abandoned in favour of animals with only rumen and duodenal cannulas. This variant is still successfully used today (Chrenková *et al.*, 2012; 2018).

This short excursion into the history of the method, although it is not directly related to the topic of this manuscript, was not purposeless. It proves that despite the attempts to avoid surgeries in experimental animals, these remain actual and necessary. In addition to limiting the demands for material and labour, all operations on animals are performed *lege artis* and in accordance to ethical principles, with the aim to minimalize the scope of surgeries, the pain through analgesics before and after the surgery and to ensure the maximum comfort of the animals during the experiments.

ACKNOWLEDGEMENTS

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PERFORMANCE OF BROILER CHICKEN FED DIETS SUPPLEMENTED WITH *IRVINGIA GABONENSIS* KERNEL POWDER AND *OCIMUM GRATISSIMUM* LEAF POWDER

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ABSTRACT

This study assessed the effects of *Irvingia gabonensis* kernel powder (IKP) and *Ocimum gratissimum* leaf powder (OLP) dietary supplementation on performance of broiler chicken. A basal diet divided into four portions, designated diet 1 (the control) and diets 2, 3 and 4 supplemented with 2.5g.kg^{-1} IKP, OLP and IKP+OLP composite mix 1:1 (IOCM), respectively. Two hundred and forty 1-day broiler chicks were randomly assigned to the four experimental diets (60 birds/diet; 10 birds per/replicate) using a Completely Randomised Design. At the finisher phase, the body weight gain and feed conversion ratio of the birds fed diets 2 and 4 were better ($P < 0.05$) than those fed the diets 1 and 3. During the overall period, the body weight gain of birds fed diet 4 was similar to those fed diet 2 but higher than those fed diets 1 and 3, while the feed conversion ratio of birds in diets 2 and 4 was better ($P < 0.05$) than those fed diets 1 and 3. The slaughtered and dressed weights of the birds fed diets 2 and 4 were significantly ($P < 0.05$) better than those birds fed the diets 1 and 3. The dressing percentage of the birds fed diets 2 and 4 were higher ($P < 0.05$) than those fed diet 1. White blood cells, granulocytes and lymphocytes counts were ($P < 0.05$) higher in birds fed diet 4 compared to those on other diets. Serum cholesterol concentration and meat lipid peroxidation activities were significantly ($P < 0.05$) lower in the birds fed diets 2, 3 and 4 compared to diet 1. The catalase concentration in the birds fed diets 3 and 4 were ($P < 0.05$) higher than those chickens fed diet 1, while the glutathione peroxidase concentration in the birds fed the diets 2 and 4 were ($P < 0.05$) higher than those fed diet 1. Glutathione concentration was higher ($P < 0.05$) in meat from birds fed diets 2, 3 and 4, compared to the birds fed diet 1. The meat cholesterol concentration recorded in the birds fed diet 4 was comparable ($P > 0.05$) to diets 2 and 3, but lower ($P < 0.05$) than diet 1. The IKP and IOCM supplementation improved the growth performance of the broiler chickens. The overall health status and meat quality were also improved by the phytogenic supplements in this study.

Key words: supplements; phytogenics; performance; anti-oxidative status; poultry

INTRODUCTION

The supplementation of diets with phytochemicals or phytogenic supplements in poultry nutrition and production has attracted considerable attention to enhancing the performance, carcass traits, health status and potentially reduce the negative effect of anti-oxidative stress since the past couple of years (Valenzuela-Grijalva *et al.*, 2017; Oloruntola *et al.*, 2018a). This is primarily because of an increased number of consumers, who are conscious of the quality and the type of their food and increased awareness of the numerous health risks associated with the use of synthetic chemicals in animal production (Gonzalez

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and Angeles, 2017). Besides, the increasing global adoption of prohibiting law against the use of antibiotic growth promoters (OJEU, 2003) further catalyses the increased hunger for searching the alternatives such as secondary plant metabolites, otherwise known as phytochemicals (Valenzuela-Grijalva *et al.*, 2017; Oloruntola *et al.*, 2019). These phytochemicals aside being considered a suitable replacement for antibiotic growth promoters are also used as other steroidal compounds (testosterone and progesterone) being used to improve the growth of animals (Gonzalez-Rios *et al.*, 2016).

Phytogenic feed supplements are a large group of compounds having diversified chemical bioavailability and structure (Surai, 2014). These phytogenic bioactive compounds in the plants vary, depending on some factors, such as the specific part of the plant, the harvest season, production techniques or methods and geographical location (Ganguly, 2013). Phytochemicals have some biological properties (antioxidant, anti-stress, antimicrobial and immunomodulatory) that prompt their consideration for use as growth promoters in livestock production (Hashemi and Davoodi, 2010).

The use of various types of phytochemicals such as extracts or parts of red pepper, lemon, clove, black cumin seed, artemisia leaf among others in broiler chicken's production produced positive results (Valenzuela-Grijalva *et al.*, 2017), while a few studies reported no significant effects (Barreto *et al.*, 2008; Goliomytis *et al.*, 2014).

Irvingia gabonensis trees produce mango-like fruits. The fruit is 4 – 7 cm long, and its kernel and pulp are edible by animals and man. The kernel is rich in oil, fat and protein and is being considered the most valuable component of the fruit (Mgbemena *et al.*, 2019). Previous phytochemical screenings of *Irvingia gabonensis* kernel reveals the presence of biologically active compounds such as tannins, alkaloids, terpenoids, steroids, saponins and glycosides, which are known for aiding antimicrobial activities (Igbinosa *et al.*, 2009; Lillehoj *et al.*, 2018; Mgbemena *et al.*, 2019).

Ocimum gratissimum is a perennial herb, which grows up to 1-2 m and having an erect stem. The *Ocimum gratissimum* plant is used in traditional medicine in India and Africa for treatment of cases, such as headache and influenza, fever, gonorrhoea, inflammation of the ears, throat or eyes, skin diseases, stomach pain and diarrhoea (Rabelo *et al.*, 2003; Adebolu and Salau, 2005; Kabir *et al.*, 2005). Prabhu *et al.*, (2002) reported the antimicrobial, antifungal, ovicidal, leishmanicidal and anti-diarrhoeal activities of *O. gratissimum* extracts.

It was observed that relatively low work was done to assess the effects of the use of *I. gabonensis* kernel and *O. gratissimum* leaf powders in broiler production compared to other medicinal plants. Also, since there could be positive effects resulting from the interactions of the various bioactive compounds in these botanicals (Brenes and Roura 2010; Oloruntola *et al.*, 2018a), this feeding trial was conducted to assess the consequences of dietary supplementation of *I. gabonensis* kernel powder, *O. gratissimum* leaf powder and their combinations on the performance, carcass traits, health status, meat analysis and antioxidant status of broiler chickens.

MATERIAL AND METHODS

Ethical approval, phytogens gathering and processing

This experiment was carried out according to the specifications and guidelines of animal and animal protocol approved by the Research and Ethics Committee of the Department of Animal Science, Adekunle Ajasin University, Akungba-Akoko, Nigeria. The pericarp, mesocarp and the endocarp of freshly plucked ripe fruits of *I. gabonensis* were removed with sharp stainless knives to expose the kernel. After that, the kernels were chopped with a stainless knife into smaller pieces, spread on a clean tarpaulin, air-dried for 21 days and ground to about 70 µm to produce *I. gabonensis* kernel powder (IKP). Freshly plucked leaves of *Ocimum gratissimum* were also chopped into smaller pieces with sharp stainless knives, spread lightly on a tarpaulin to air-dry for 14 days and milled to the particulate size of 70 µm to produce *O. gratissimum* leaf powder (OLP). Equal portions (1:1) of IKP and OLP were mixed to form *I. gabonensis* and *O. gratissimum* leaf powder composite mix (IOCM). After that, the IKP, OLP, and IOCM were analysed for saponin (Brunner, 1984), flavonoids (Bohm and Kocipal-Abyazan, 1994), phenol (Ignat *et al.*, 2013), terpenoids (Sofowora 1993) and 2,2-diphenyl-1-picrylhydrazy hydrate (Gyamfi *et al.*, 1999).

Diets, housing and experimental design

A broiler chickens' basal diet each was prepared for the starter phase (0 to 28 days) and finisher phase (29-56 days) to meet the requirements of the birds (NRC, 1994). At each of the phases, the basal diet was divided into four equal portions and designated to Diets 1 to 4. Diet 1 was the control, while the diets 2, 3 and 4 were supplemented with 2.5 g of IKP, OLP and IOCM.kg^{-1}, respectively. The experiment was performed at the Avian Unit of the Teaching and Research Farm, Adekunle Ajasin University, Akungba-Akoko, Nigeria.

Two hundred and forty (240) 1-day old Cobb 500 broiler chicks with an average initial body weight of 44.99 ± 0.90 g were randomly assigned to four experimental diets (60 birds per diet; 10 birds per replicate) using a completely randomised design (CRD). The floor of the experimental pen (200 x 100 cm) used for housing each replicate was covered with wood shaving while the temperature of the experimental house was maintained at 31 ± 2 °C for the first week and gradually being reduced by 2 °C after each consecutive week until the experimental house temperature was 26 ± 2 °C. The lighting duration was 23 hours per day, while the feed for the birds was provided *ad libitum* throughout the experiment.

Growth performance

The experimental birds' body weight (BW) and feed intake (FI) were determined and recorded on a 7-day interval. The average body weight gain (BWG) was calculated as the differences between the initial weights and final weights of the birds while their feed conversion ratio (FCR) was estimated as the ratio of feed consumed to weight gain.

Slaughtering procedures, collection of blood samples and carcass analysis

On day 56 of the experiment, 18 birds randomly selected from each dietary treatment (3 birds/replicate) were tagged, weighed, stunned and sacrificed by cutting the two jugular veins in the neck region with a stainless-steel knife. Blood was allowed to flow into a plain blood sample bottle for serum biochemicals and enzymes (creatinine, aspartate aminotransferase, alanine aminotransaminase, and cholesterol); antioxidant enzymes (catalase, superoxide dismutase and, glutathione peroxidase) and also into EDTA bottle for haematological studies. The blood sample in each of the plain bottles was spun and its serum decanted into another plain bottle and frozen at -20 °C before analysis. The haematological indices were determined within 2 hours post-collection as described by Shastry (1983). The concentrations of serum enzymes were determined on a Reflectron ®Plus 8C79 (Roche Diagnostic, GombH Mannheim, Germany), using kits. The serum catalase, superoxide and glutathione peroxidase were determined as described by Aebi (1974), Misra and Fridovich, (1972) and Rotruck *et al.*, (1973), respectively.

The selected slaughtered experimental birds were de-feathered, dressed and weighed. After that, the dressed percentage was estimated as a percentage of the slaughtered weight. The internal organs (liver, heart, lung, pancreas, gall bladder, gizzard and proventriculus, and the spleen) were carefully excised, wiped clean with tissue paper and weighed with a sensitive scale. The relative internal organ weight was expressed as a percentage of the bird's slaughtered weight. About 100 g of the meat was excised from the breast meat for determination of the level of the meat cholesterol (Allain *et al.*, 1974), lipid peroxidation (Bostoglou *et al.*, 1994), catalase activity (Hadwan and Khabt, 2018) and glutathione peroxidase activity (Cichoski *et al.*, 2012).

The contents of the caeca from the experimental birds (1 bird/replicate) were collected for bacterial population's analysis by serial dilution. Agar plates were aseptically prepared a day before the caecal content collection. The plates were streaked on the experimental site to determine the bacteria's growth. The aerobic bacteria were cultured in the nutrient agar, lactic acid-producing bacteria were cultured on Man Rogosa agar, while the coliforms and intestinal negative lactose bacteria were cultured in the MacConkey agar (Dibaji *et al.*, 2014; Seidavi and Simoes 2015).

Analysis of data

The model: $Txy = \mu + \alpha x + \beta xy$, was used in this experiment, where Txy = any of the response variables; x = the overall mean; αx = effect of the *xth* treatment (T = diets 1, 2, 3 and 4); and βxy = random error due to experimentation. All the data were subjected to one-way ANOVA using SPSS version 20. The differences among the treatment means were determined ($P < 0.05$) by Duncan multiple range test of SPSS.

RESULTS

The contents of saponin (34.74 mg.g^{-1} vs. 26.08 mg.g^{-1}), flavonoid (3.19 mg.g^{-1} vs. 0.26 mg.g^{-1}), phenol (19.61 mg.g^{-1} vs. 14.97 mg.g^{-1}) and terpenoid (94.18 mg.g^{-1} vs. 89.42 mg.g^{-1}) in IKP- versus OLP are presented in Figure 1. The concentrations of the phytochemicals determined were higher in IKP- compared to OLP. Figure 2 shows the antioxidant property (2,2-diphenyl-1-picrylhydrazyl hydrate) of IKP- (28.10 %) and OKM- (35.50 %).

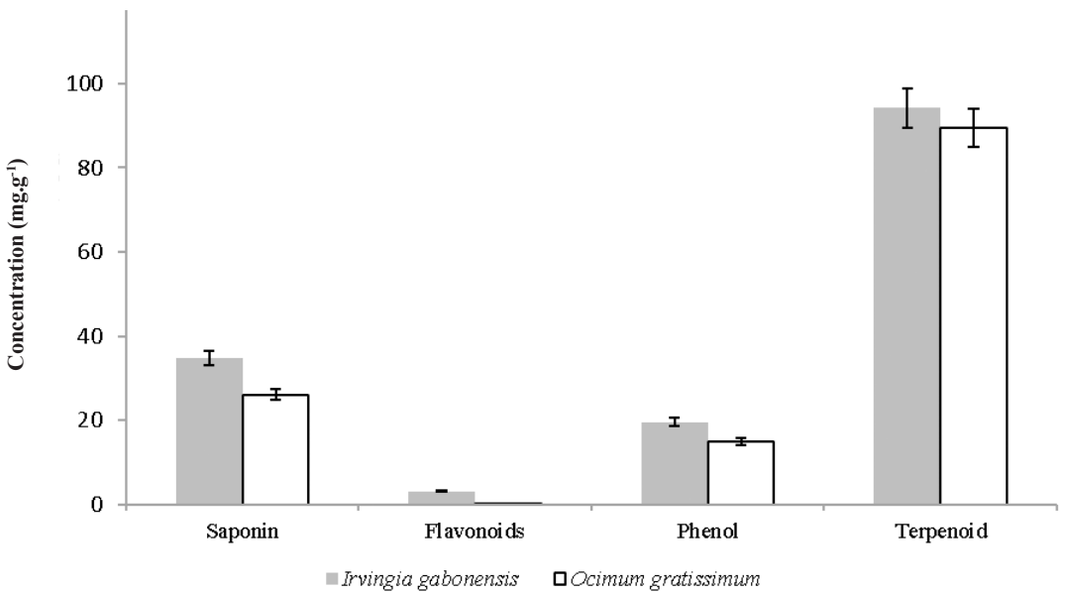

Figure 1. Phytochemical compositions of *I. gabonensis* kernel and *O. gratissimum* leaf powders

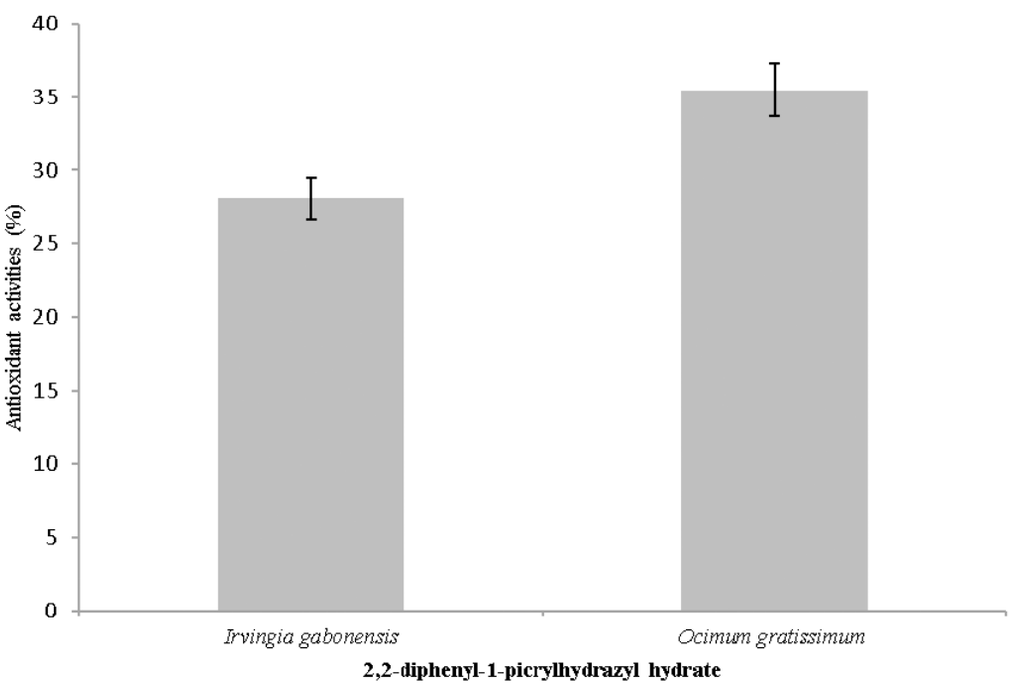

Figure 2. Antioxidant activities of *I. gabonensis* kernel and *O. gratissimum* leaf powders

Table 1. Composition of the experimental diets

Ingredients	Starter diet	Finisher diet
Maize	52.35	59.35
Maize bran	7.00	0.00
Rice bran	0.00	6.00
Soybean meal	30.00	24.00
Fish meal	3.00	3.00
Soy oil	3.00	3.00
Bone meal	3.00	3.00
Limestone	0.50	0.50
Lysine	0.25	0.25
Methionine	0.30	0.30
Salt	0.30	0.30
Premix	0.30	0.30
Nutrient composition (%)		
*Crude protein	22.18	20.03
Metabolizable energy (Kcal.kg^{-1})	3018.89	3108.10
Lysine	1.36	1.24
Methionine	0.68	0.66
Calcium	1.01	0.99
Available phosphorus	0.70	0.73

*Analysed composition

The effects of the phytogenic supplementations on the body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) during the starter phase (1-28 day), finisher phase (29-56 day) and overall (1-56 day) are presented in Table 2. During the finisher phase, the BWG and FCR of the birds fed IKP and composite mix of IKP and IOCM supplemented diets (Diets 2 and 4) were similar ($P > 0.05$), but significantly ($P < 0.05$) better than those birds fed the control diet and OLP-supplemented diet. For the overall period of the experiment, the BWG of the birds fed diet 4 was similar to those fed diet 2, but significantly ($P < 0.05$) higher than those fed the diets 1 and 3. The FCR of the birds fed diets 2 and 4 were similar ($P > 0.05$) to those fed diet 3, but better ($P < 0.05$) than those fed diet 1. The slaughtered and dressed weights of the birds fed IKP- and IOCM-supplemented diets were higher ($P < 0.05$) compared to the control diet and OLP-supplemented diet (Table 3). Similarly, the dressing percentage of the birds fed diets 2 and 4 were similar ($P > 0.05$) to those fed diet 3, but higher ($P < 0.05$) than those fed the control diet.

The significant dietary treatment effects were not recorded for the haematological indices except for the white blood cell (WBC), granulocyte and lymphocyte counts that were significantly ($P < 0.05$) higher in birds fed diet 4 compared to those fed the other diets (Table 4). Table 5 shows the effects of the phytogenic supplements on the serum metabolites

Table 2. Effects of the phyto-supplements on the performance characteristics of broiler chickens

Parameters	Diet 1 Control	Diet 2 0.25 % IKP	Diet 3 0.25 % OLP	Diet 4 0.25 % IOCM	SEM	*P*-value
Starter phase (1 to 28 day)						
IBW (g/bird)	43.09	47.52	45.42	43.93	0.90	0.35
BW G (g/bird)	990.10	1052.60	1040.01	1156.22	34.86	0.44
FI (g/bird)	1407.46	1307.15	1406.66	1412.30	35.91	0.74
FCR	1.44	1.27	1.35	1.22	0.06	0.63
Finisher phase (29 to 56 day)						
BWG (g/bird)	1824.30^{b}	2161.11^{a}	1687.26^{b}	2218.98^{a}	79.85	0.01
FI (g/bird)	3561.04	3238.67	3250.77	3532.37	77.07	0.30
FCR	1.95^{a}	1.51^{b}	1.93^{a}	1.59^{b}	0.07	0.01
Overall (1 to 56 day)						
BWG (g/bird)	2314.40^{b}	3213.71^{ab}	2727.27^{b}	3374.20^{a}	103.65	0.04
FI (g/bird)	4968.51	4545.83	4657.43	4944.68	101.17	0.40
FCR	1.77^{a}	1.42^{b}	1.71^{ab}	1.46^{b}	0.05	0.05

Means within a row with different letters are significantly different ($P < 0.05$); IKP: Irvingia kernel powder; OLP: Ocimum leaf powder; IOCM: Irvingia and Ocimum composite mix (1:1); SEM Standard error of the mean.

Table 3. Effects of phyto-additives on carcass and relative internal organ weights (% slaughtered weight) of broiler chickens

Parameters	Diet 1 Control	Diet 2 0.25 % IKP	Diet 3 0.25 % OLP	Diet 4 0.25 % IOCM	SEM	*P*-value
Slaughtered weight (g/bird)	2618.66^{b}	3254.67^{a}	2570.65^{b}	3436.01^{a}	115.34	0.01
Dressed weight (g/bird)	2016.65^{b}	2630.34^{a}	2118.02^{b}	2795.65^{a}	107.52	0.01
Dressing percentage (%)	79.95^{b}	80.78^{a}	79.34^{ab}	81.31^{a}	0.66	0.04
Liver	1.62	1.46	1.61	1.33	0.05	0.19
Heart	0.41	0.34	0.34	0.35	0.01	0.46
Lung	0.47	0.42	0.44	0.40	0.22	0.82
Pancreas	0.16	0.11	0.16	0.13	0.01	0.67
Gall bladder	0.13	0.09	0.16	0.10	0.01	0.13
Gizzard and proventriculus	2.37	1.96	2.09	1.94	0.09	0.42
Spleen	0.13	0.11	0.10	0.08	0.01	0.50

Means within a row with different letters are significantly different ($P < 0.05$); IKP: Irvingia kernel powder; OLP: Ocimum leaf powder; IOCM: Irvingia and Ocimum composite mix (1:1); SEM Standard error of the mean.

Table 4. Effects of phyto-additives on haematological indices of broiler chickens

Parameters	Diet 1 Control	Diet 2 0.25 % IKP	Diet 3 0.25 % OLP	Diet 4 0.25 % IOCM	SEM	*P*-value
Packed cell volume (%)	33.66	33.50	33.50	37.90	0.81	0.06
Red blood cells (x10^{12}.l^{-1})	3.00	2.90	2.30	3.00	0.18	0.53
Haemoglobin conc. (g.dl^{-1})	11.46	11.16	11.16	12.55	0.23	0.10
Mean cell haemoglobin conc. (g.dl^{-1})	35.19	33.72	33.23	33.37	0.32	0.10
Mean cell volume (fl)	110.53	123.48	149.61	133.90	8.01	0.40
Mean cell haemoglobin (pg)	39.02	41.16	49.87	40.36	2.75	0.55
White blood cells (x10^{9}.l^{-1})	3.90^{b}	2.60^{c}	3.40^{bc}	7.23^{a}	0.55	0.00
Granulocytes (x10^{9}.l^{-1})	0.74^{c}	0.51^{c}	1.67^{b}	2.97^{a}	0.30	0.00
Lymphocytes (x10^{9}.l^{-1})	3.10^{b}	2.05^{c}	1.64^{c}	4.17^{a}	0.30	0.00
Monocytes (x10^{9}.l^{-1})	0.04	0.03	0.08	0.06	0.01	0.57

Means within a row with different letters are significantly different ($P < 0.05$); IKP: Irvingia kernel powder; OLP: Ocimum leaf powder; IOCM: Irvingia and Ocimum composite mix (1:1); SEM Standard error of the mean.

and enzymes of the broiler chickens. The serum creatinine, aspartate aminotransferase and alanine aminotransferase were not affected ($P > 0.05$) by the dietary treatment, while the serum cholesterol concentrations were significantly ($P < 0.05$) lower in birds fed diets 2, 3 and 4 compared to those fed the diet 1. The serum catalase concentrations in the birds fed diets 3, and 4 were significantly ($P < 0.05$) higher than in those chickens fed the control diet, while the highest serum glutathione peroxidase concentration recorded in the birds fed diets 2 and 4 was comparable ($P > 0.05$) to those fed the diet 3 but significantly ($P < 0.05$) higher than those fed the diet 1.

The effects of phytogenic supplements on the lipid peroxidation, antioxidant enzymes and cholesterol of the meat were shown in Table 6. The lipid peroxidation activities were significantly ($P < 0.05$) reduced in diets 2, 3 and 4, so that the least lipid peroxidation was recorded in meat from the birds fed diet 4. The catalase activity was not affected ($P > 0.05$) by dietary treatment. However, the glutathione concentration was higher ($P < 0.05$)

Table 5. Effects of phyto-additives on serum metabolites and serum antioxidant enzymes of broiler chickens

Parameters	Diet 1 Control	Diet 2 0.25 % IKP	Diet 3 0.25 % OLP	Diet 4 0.25 % IOCM	SEM	*P*-value
Serum metabolites						
Creatinine (μmol.L^{-1})	43.48	47.33	29.00	35.16	6.83	0.82
Aspartate aminotransferase (IU.L^{-1})	75.82	108.66	89.00	83.56	5.98	0.26
Alanine aminotransferase (IU.L^{-1})	28.67	30.05	31.96	28.84	1.28	0.83
Cholesterol (μmol.L^{-1})	6.68^{a}	3.07^{b}	3.15^{b}	3.13^{b}	0.55	0.02
Serum antioxidant enzymes						
Catalase (mM.ml.min^{-1})	5.90^{c}	9.09^{bc}	17.40^{a}	12.40^{ab}	1.51	0.01
Superoxide dismutase (%)	75.38	75.64	74.46	61.93	3.99	0.62
Glutathione peroxidase (μg.g^{-1})	77.68^{b}	133.16^{a}	119.56^{ab}	145.36^{a}	9.76	0.04

Means within a row with different letters are significantly different ($P < 0.05$); IKP: Irvingia kernel powder; OLP: Ocimum leaf powder; IOCM: Irvingia and Ocimum composite mix (1:1); SEM Standard error of the mean.

Table 6. Effects of phyto-additives on the quality of broiler chicken meat

Parameters	Diet 1 Control	Diet 2 0.25 % IKP	Diet 3 0.25 % OLP	Diet 4 0.25 % IOCM	SEM	*P*-value
Lipid oxidation (mg MDA.100 g^{-1})	13.72^{a}	7.94^{b}	7.39^{bc}	3.43^{c}	1.24	0.00
Catalase (U.ml^{-1})	1.93	2.05	1.38	2.87	0.28	0.35
Glutathione peroxidase (mg.ml^{-1})	149.83^{b}	253.50^{a}	227.59^{a}	247.08^{a}	15.11	0.02
Cholesterol (mg.dl^{-1})	218.75^{a}	138.12^{ab}	106.25^{ab}	43.75^{b}	23.72	0.03

Means within a row with different letters are significantly different ($P < 0.05$); IKP: Irvingia kernel powder; OLP: Ocimum leaf powder; IOCM: Irvingia and Ocimum composite mix (1:1); SEM Standard error of the mean.

Table 7. Effects of the phyto-supplements on intestinal microbiology (log10 CFU.g^{-1}) of broiler chickens

Parameters	Diet 1 Control	Diet 2 0.25 % IKP	Diet 3 0.25 % OLP	Diet 4 0.25 % IOCM	SEM	*P*-value
Aerobic bacteria	5.05	4.98	5.35	5.05	0.14	0.84
Lactic acid-producing bacteria	5.80	5.79	5.38	5.53	0.07	0.13
Coliform bacteria	5.13	4.75	4.73	4.51	0.15	0.64
Intestinal negative bacteria	5.05	4.68	4.81	5.43	0.14	0.28

Means within a row with different letters are significantly different ($P < 0.05$); IKP: Irvingia kernel powder; OLP: Ocimum leaf powder; IOCM: Irvingia and Ocimum composite mix (1:1); SEM Standard error of the mean.

in meat from birds fed diets 2, 3 and 4, compared to the meat from the birds fed the diet 1. The least meat cholesterol concentration recorded in the meat of the birds fed the diet 4 was comparable ($P > 0.05$) to those fed diets 2 and 3, but significantly ($P < 0.05$) lower than those fed the diet 1. The phytosupplements did not cause significant difference ($P > 0.05$) in the aerobic bacteria, lactic-acid producing bacteria, coliform bacteria and intestinal negative bacteria populations in the chickens' intestine (Table 7).

DISCUSSION

In this study, the presence of secondary bioactive compounds such as saponin, flavonoids, phenol and terpenoid and the antioxidant activities of IKP and OLP suggests that these phytogenics, when incorporated into the diets of the broiler chickens, could generate beneficial effects on their performance and health status.

The variability of the birds' response to the dietary treatment between the starter and finisher phases of the birds suggests that the age and another factors, such as differences in the nutritional requirements and management practices between the two phases, may influence the growth response of these birds to dietary phyto-supplementation. This is in agreement with Oloruntola *et al.* (2018a), who recorded the effects of phyto-supplements on the growth performance of the broiler chicken at the finisher phase but not at the starter phase. According to Valenzuela-Grijalva *et al.* (2017), most of the *in vivo* studies evaluating the effects of dietary phyto-supplementation on the growth performance of broiler chickens were positive. In this study, the observed improved BWG and FCR of the experimental birds fed IKP- and IOCM-supplemented diets during the finisher phase (29-56 day) and overall (1-56 day) could be due to the activities of the constituents of these phytogenics. The biological activities (antimicrobial, antioxidant and flavour enhancer) of phytochemicals were reported (Negi, 2012; Valenzuela-Grijalva *et al.*, 2017) and could have contributed to the improved BWG and FCR recorded in these groups of birds. Besides, phytochemicals were reported to exert anabolic effects and modulate the animals' metabolism to influence the increase of the muscle tissue (Devi *et al.*, 2015; Gonzalez-Rios *et al.*, 2016). In particular, the flavonoid, one of the phytoconstituents of IKP and OLP is known for its active role in the suppression of reactive oxygen species (ROS) formation, scavenging for ROS and protection and upward regulation of antioxidant defences (Halliwell and Gutteridge, 1998; Mishra *et al.*, 2013). Flavonoid is also known as an established hepatoprotective, anticancer, anti-inflammatory and antiviral agent (Kumar and Pandey, 2013). Therefore, the antioxidant activities of the phytogens used in this study may also contribute to the enhanced weight gain and feed conversion ratio recorded in the birds fed IKP- and IOCM-supplemented diets in this study.

Lillehoj *et al.* (2018) reported that a combination of multiple phytogenics exerted synergistic effects to ameliorate the adverse consequences of intestinal infection. This could be responsible for the relatively superior growth performance observed in the birds fed with IOCM-supplemented diet in this study. Since the phyto-supplementation did not exert any effect on the feed intake in the experimental birds across the various dietary treatments in this study, the enhanced growth performance recorded in the birds fed IKP- and IOCM-supplemented diets may be due to the activities of the phytoconstituents of IKP and the synergistic effects IOCM to stimulate the functions of the intestinal tract to improve the digestive secretion, nutrient digestion, absorption and metabolism. Phytochemicals exhibit biological activities, such as a decrease of pathogenic load, increase of digestive secretions, development of antioxidant and anti-inflammatory activities in the intestinal lumen and improved intestinal morphology, which may result in improved nutrient utilisation and enhanced growth (Dhama *et al.*, 2014).

The improved slaughtered weight dressed weight, and dressed weight percentage recorded in the birds fed the IKP- and IOCM-supplemented diets in this study agreed with the earlier reports that supplementation of the broiler chickens diet with phytochemicals such as thyme, lemon balm, essential oil blend and cinnamon improved the carcass weight and dressed percentage (Kanduri *et al.*, 2013; Valenzuela-Grijalva *et al.*, 2017). This suggests that the phytogenic supplements used in this study have phytoconstituents or bioactive compounds (e.g., hydroxycinnamic acid derivatives of the phenylalanine) that can modulate animal metabolism in a similar pattern with β-adrenergic agonist compound (Gorewit, 1983; Valenzuela-Grijalva *et al.*, 2017). These plant-based compounds have a similar structure with the catecholamines (the natural animal hormones). They could interact with β-adrenergic receptor agonists to modulate animal metabolism by increasing lipolysis and protein synthesis and by decreasing lipogenesis (Dominguez-Vira *et al.*, 2009). The decrease or increase in the relative weights of the internal organs of the animals has been reported as a possible

response of their internal organs to toxins in their diets (Ayodele *et al.*, 2016). The similarity in the growth response of these animals' internal organs to the phytogenic supplementation in this study suggests the support of the supplements to the normal functioning of the birds' internal organs.

There is a relationship existed between nutritional deficiency and changes in the blood constituents. The haematological indices are among the known good indicators of the physiological status of the animals (Khan and Zafar, 2005). In this study, the stable erythrogram constituents' values recorded in the birds across the various dietary treatments indicate that the dietary phyto-supplements did not have a harmful interference on the haematopoiesis in the experimental birds. The variations recorded in the white blood cell values and their differentials across the dietary treatments in this study may indicate the immunomodulatory effects of the phytogenic supplements in the experimental birds, as reported by Oloruntola *et al.* (2016). The white blood cells play a significant role in the fighting against an infection. Therefore, the rise in the white blood cell and the differential counts (granulocytes and lymphocytes) of the birds fed the IOCM-supplemented diets in this study suggests that the IOCM (i.e. the composite mix of IKP and OLP) could trigger a complex but beneficial immunomodulatory response in the birds. This is supported by the earlier report of Hang and Lee (2018). The phytochemicals such as flavonoids, carotenoids and vitamin C have been shown to possess immune-stimulatory properties by improving the activities of lymphocytes, monocytes, macrophages, the immunoglobulin response and NK cells (Frankic *et al.*, 2009; Alipour *et al.*, 2015).

The similar creatinine concentration in the birds fed phytogenic-supplemented diets and the control suggest that the supplements used in this study did not pose any threat to the renal functions of the birds (Peters and Susan, 1991). The aspartate aminotransferase and alanine aminotransferase levels are commonly used to detect hepatic cell infraction and inflammation (Oloruntola *et al.*, 2018a, b). The stable aspartate aminotransferase and alanine aminotransferase concentrations negate the occurrence of the liver and biliary system disease, skeletal muscle disease, myocardial disease and non-specific tissue injury in the experimental birds as a result of the phytogenic supplementation tested in this study (Peter and Susan, 1991). Excess of serum cholesterol concentration promotes cholesterol accumulation on the artery walls creating plaques that lead to the narrowing of the arteries lumen and reduction of the rate of blood flow to the heart (Oloruntola *et al.*, 2018a, c). Therefore, the observed reduced serum cholesterol concentration recorded in the birds fed the phytogenic-supplemented diets in this study is of health benefit because abnormally high serum cholesterol concentration has an association with arteriosclerosis and sudden death syndrome in broiler chickens (Kawada *et al.*, 1994; Olkowski *et al.*, 2007) what also suggests IKP, OLP and IOCM possessed hypo-cholesterol properties.

The observed reduced cholesterol level, as a result of phytogenic supplementation in this study, may be due to the activities of secondary metabolites (e.g. saponin) in IKP, OLP and IOCM, that promoted the reduction of the gut uptake of cholesterol through the intra-luminal physicochemical interaction (Oloruntola *et al.*, 2018a). Also, phytochemicals or phytogens inhibit 3-hydroxy-3methylglutarycoenzyme A (HMG Co-A) reductase; and the consequence of this inhibition may be pleiotropic, because mevalonate, the product of HMG Co-A reductase reaction is the precursor for cholesterol (Bellosta *et al.*, 2000; Vaughan *et al.*, 2000). Enzymatic activities, such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), provide protection against oxidative stress (Young and Woodside, 2001). The relatively higher catalase and glutathione peroxidase activities recorded in the birds fed the IKP-, OLP- and IOCM- supplemented diets in this study further unveiled the antioxidant and anti-stress properties of these phytogenics (Hashemi and Davoodi, 2010). The higher antioxidant activities recorded in the birds fed the IKP-, OLP- and IOCM-supplemented diets in this work may be due to the polyphenolic contents (flavonoids or phenolic acids) of the phytogens (Goyal and Brahma, 2014). According to Dhama *et al.* (2015), the plants' active ingredients generate strong antioxidant effects that scavenge the radicals or increase the CAT, SOD and GPx activities. The antioxidant activities of these phytogenics may also have contributed to the superior performance recorded in those birds fed

the phytogenic-supplemented diets, compared to those fed the control diet in this study. According to Biswas *et al.* (2011), antioxidants play a significant role in the performance of poultry.

Oxidative process during the shelf-life of meat can depreciate its nutritional and sensory values (Kumar *et al.*, 2015). Therefore, strategies that can promote the avoidance of the oxidation of lipids and proteins will contribute to the extension of the useful life/shelf life of meat (Velasco and Williams, 2011). This is because the progression of lipid oxidation promotes the loss of physiological function, membrane property alteration, enzyme inactivation, denaturation and rupture, causing cellular component leakage (Bekhit *et al.*, 2013). Currently, the dietary inclusion of natural antioxidants during animal production is being proposed (Brewer, 2011) to forestall the deposition of antioxidants in the meat during the life of the animals and subsequently enhancing the health status of the animals and the shelf life of the meat by enhancing the oxidative status ante-mortem (Descalzo and Sancho, 2008).

The reduced lipid peroxidation activities in the meat of the birds fed the IKP-, OLP- and IOCM-supplemented diets in this study further support the fact that dietary inclusion of phytogenics in the animals' diet during production could reduce the lipid peroxidation activities and subsequent improvement of the meat shelf life (Descalzo and Sancho, 2008, Valenzuela-Grijalva *et al.*, 2017). Peroxide (H_2O_2) can diffuse within the cell and produce noticeable damages to the cells and the muscle systems of living organisms (Bekhit *et al.*, 2013). Therefore, the availability of catalase, thioredoxin peroxidase and glutathione peroxidase is essential in eliminating H_2O_2 (Marchi *et al.*, 2012). The higher glutathione peroxidase concentration recorded in the meat from birds fed the IKP-, OLP- and IOCM-supplemented diets in this study unveiled the possibility of these phytogenic supplements to modify the antioxidant enzymes in the muscular system and subsequent increase of the meat shelf life (Bekhit *et al.*, 2013). Presently, the type of cholesterol and fatty acids are of health importance to the consumers because of the existing relationship between the consumption of high cholesterol and saturated fat and an increased possibility of acquiring diseases such as high blood pressure, cancer, heart disease and obesity (Walker *et al.*, 2005). The reduced meat cholesterol recorded in birds fed IKP-, OLP- and IOCM-supplemented diets compared to those fed the control diet in this study may have an association with the low blood serum recorded in the same group of birds. There is a need to research further if there is a relationship exists between the blood and meat cholesterol concentrations. However, the reduced meat cholesterol level recorded in these birds, is of health benefit because of the enormous health complication associated with the ingestion of high cholesterol meat.

The gut of broiler chickens is densely colonised by the community of microorganisms that is intimately linked to the general health and development of the host (Oakley *et al.*, 2014). The avian caeca housed microorganism that functions in the breaking down of indigestible fibre substance and maintenance of the health status of the birds (Zulkifli *et al.*, 2009; Oakley *et al.*, 2014). For instance, lactic acid bacteria (probiotic microorganism), are usually associated with the maintenance and enhanced gut health and productivity because of their activities in reducing enteric diseases (Noohi *et al.*, 2014). Also, controlled adhesion of antimicrobial phytogens and subsequent modulation of the gut microflora promotes the maintenance of the intestinal epithelium integrity, reduced toxin production and increased nutrient availability for absorption (Dhama *et al.*, 2014). The observed similarity in the intestinal microbial population of the broiler chickens in this study implies the phytogenic supplementations used, which are able to maintain the healthy and stable intestinal microflora. A stable increase of non-pathogenic gut bacteria was reported as a necessary condition for inhibition of the proliferation of pathogens, improved growth performance and production, reduced morbidity and mortality (Gou *et al.*, 2004; Oloruntola *et al.*, 2020).

CONCLUSION

The IKP and OLP have phytochemicals of health benefits and possess antioxidant properties. The IKP and IOCM at 0.25 % dietary supplementation improved the BWG, FCR, slaughtered weight and dressed weight of broiler chickens at the finisher

phase. The phytogenic supplementation also caused immunomodulatory and hypocholesterolemic effects on the broiler chickens. The IKP, OLP and IOCM at 0.25 % dietary supplementation reduced the lipid peroxidation, increased the glutathione concentration and reduced the cholesterol level of the breast meat of the broiler chickens.

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GROWTH PERFORMANCE, BLOOD INDICES AND INTESTINAL ORGAN DEVELOPMENT OF PULLET CHICKS ADMINISTERED AQUEOUS EXTRACTS OF GUINEA HEN WEED (*PETIVERIA ALLIACEA*)

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ABSTRACT

This study investigated growth performance, intestinal organ development and blood indices of pullet chicks administered aqueous extracts of *Petiveria alliacea* root and leaves. A total of 288 day-old Isa brown pullets were randomly allotted into two groups administered aqueous extract of different parts of *Petiveria alliacea* (root and leaf) at four concentration levels (0, 15, 30 and 45 $g.l^{-1}$) making a total of 8 treatments. Each treatment was replicated three times with 12 birds per replicate. The experiment was arranged in a 2 × 4 factorial experimental layout in a completely randomized design. There were similarities ($P > 0.05$) in feed intake, feed conversion ratio, weight gain and final weight across all treatments. Lowest ($P < 0.05$) water intake was recorded in pullet chicks maintained on 30 $g.l^{-1}$ leaf extract (2726.90 ml/bird). Birds maintained on 30 $g.l^{-1}$ and 45 $g.l^{-1}$ concentration of extraction had the highest ($P < 0.05$) caeca weight (0.75 % of body weight). Birds administered root extract and leaf extract at 30 g. l^{-1} and 45 $g.l^{-1}$ concentration levels recorded higher ($P < 0.05$) white blood cell count compared to other treatments. Highest ($P < 0.05$) lymphocyte differential was recorded in birds raised on 45 $g.l^{-1}$ root extract. Lowest ($P < 0.05$) serum uric acid was recorded in pullet chicks administered 45 $g.l^{-1}$ root extract (3.07 $mg.dl^{-1}$), while serum cholesterol was lowered ($P < 0.05$) in all administered levels of extracts of *Petiveria alliacea* when compared to the control birds. The study concluded that administration of aqueous root extract of *Petiveria alliacea* at 45 $g.l^{-1}$ concentration level best enhanced immunity, reduced serum urea and cholesterol in pullet chicks without impairing their growth.

Key words: growth performance; blood indices; pullet chicks; *Petiveria alliacea* root; *Petiveria alliacea* leaf

INTRODUCTION

Poultry production represents one of the largest sub-sectors of the Nigerian agricultural industry (Bamiro *et al.*, 2006). Gradual growth of the poultry industry can be attributed to systemic and timely application of innovative sciences (Alabi and Samuel, 2002).

Commercial layers capable of producing remarkable amount of eggs during their life-time has been developed through improvement in genetic make-up, modification of diet and improvement

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in management practices (Alabi and Samuel, 2002). The major aim of egg-type chicken production is to raise a disease-free stock to attain optimal body weight and egg production in return for each unit of feed intake (Asrat *et al.*, 2018). According to Holik (2015), the basis for good egg production is a successful rearing phase. This means that management, health and growth performance of pullet chicks at early age, despite accounting for a small portion of the bird's life, have significant impact on subsequent egg production performance. Poor nutrition at early age was reported to limit growth potential (Henderson *et al.*, 2008) and induce poor performance as a result of increased susceptibility to diseases in chickens (Noy and Sklan, 1999; Dibner *et al.*, 1998). For commercial egg-type chickens having the genetic potential to rapidly attain peak of egg production, good body reserves and health status is necessary to achieve satisfactory performance in the laying house (Summers, 2008). Therefore, appropriate health maintenance practices and proper feeding management are necessary right from the early stage of growth to produce healthy and excellent egg-producing laying stock.

Blood is a key index in evaluating physiological, pathological and nutritional state of avian species (Hauptmanova *et al.*, 2006; Olorode *et al.*, 2007). Dietary intake has been identified as a major factor affecting blood composition (Aletor, 1989; Aletor and Egberongbe, 1992). Relative comparison of blood constituent compositions with normal standardized values could be used to predetermine the metabolic status of an animal as well as feed utilization and quality (Babatunde *et al.*, 1992).

Antibiotics have been used in poultry production as growth promoters and for therapeutic purposes against many diseases facing the poultry industry. However, recently the use of antibiotic drugs has been restricted due to emergence of antibiotic resistant microorganisms and harmful drug residues in food products (Castanon, 2007; Diarra and Malouin, 2014; Dhama *et al.*, 2015). Also, increasing awareness of the public on food safety has led to high demand of antibiotic-free food products of poultry sources (Biswas *et al.*, 2010). These trends necessitated researchers to find sustainable alternatives to the use of antibiotics for general health maintenance, immunomodulation, growth enhancement and therapeutic purposes (Diarra and Malouin, 2014). The use of herbal plant has emerged as one of the viable alternatives to antibiotic drugs (González-Lamothe *et al.*, 2009). Considering feed efficiency, weight gain and improved liveability in poultry production, the use of herbal plants have shown some promising results (Mishra and Singh 2000; Deepak *et al.*, 2002; Jahan *et al*., 2008).

Petiveria alliacea belonging to the plant family *phytolaccacea* is a perennial herb that grows widely in tropical regions (Lopes-Martins *et al.*, 2002). The root and leaves has been used in folk medicine against various diseases (Kim *et al.*, 2006) and as antispasmodic, sedative, diuretic, anti-helminthic, menstrual promoting, stimulant, analgesic, anti-inflammatory and anti-cancerous agent (Lopes-Martins *et al.*, 2002). *Petiveria alliacea* has also been reported to possess broad range of antimicrobial properties (Illnait-Zaragozí *et al.*, 2014; Ekunseitan *et al.*, 2016). Phytochemical screening of the plant showed presence of Terpenoid, Flavonoid, Tannin, Alkaloids, Phytate, Phenols, Antioxidant, Saponin, Oxalate and Carotenoids (Ekunseitan *et al.*, 2016). Few studies have been conducted recently, investigating the growth response of growing pullets (Muhammad *et al.*, 2018) and broiler chickens (Odetola *et al.*, 2019) to *Petiveria alliacea* root and leaf meal. However, the potential of extracts from fresh roots and leaves of *Petiveria alliacea* on growth performance and health of poultry species is yet to be explored. Therefore, this study was conducted to evaluate the growth performance, intestinal organ development and blood indices of pullet chicks administered aqueous extract of *Petiveria alliacea*.

MATERIAL AND METHODS

Ethical Statement

This study was performed in accordance with the ethical guidelines of Animal Welfare Committee, College of Animal Science and Livestock Production, Federal University of Agriculture, Abeokuta, Nigeria.

Experimental Site

The experiment was conducted at the Poultry Unit of Livelihoods Support and Development Centre (SLIDEN AFRICA), Alabata road, Abeokuta, Ogun State, Nigeria.

Harvesting and Extraction of *Petiveria alliacea*

Fresh roots and leaves of *Petiveria alliacea* were harvested from Kotopo Area of Abeokuta, Nigeria. They were rinsed thoroughly with clean water to remove sand and other dirt. Aqueous extraction of *Petiveria alliacea* was done as described by Nodu *et al.* (2016). 15 g, 30 g and 45 g of roots and leaves were weighed individually and each was blended separately in 1 litre of water for 120 seconds. After blending, each mixture was filtered to remove root and leaf particles using appropriate sieve. The filtrates were supplied to birds separately as drinking water according to the treatment groups. Volume of filtrates supplied to birds at all period of administration was enough to meet and exceed daily water requirement of the birds.

Table 1. Percentage composition of experimental diet

Ingredients	%
Maize	50.00
Soya bean meal	10.00
Fish meal (72 % CP)	1.70
Groundnut cake	20.00
Wheat offal	15.00
Oyster shell	1.00
Bone meal	1.50
Lysine	0.15
Methionine	0.15
Common salt	0.25
Premix	0.25
Total	100.00
Determined Analysis (%)	
Dry matter (%)	88.70
Crude Protein (%)	21.80
Crude Fibre (%)	5.00
Ether extracts (%)	3.74
Ash (%)	6.00
Metabolisable energy (MJ.kg^{-1})	11.91

Premix (Composition per kg diet): Vit. A (I.U.) 2,800,000; Vit. E (mg) 16,000; Vit. K (mg) 800; Vit. B_1 (mg) 1,200; Vit. B_2 (mg) 1,600; Vit. B_6 E.E4 (mg) 30; Folic Acid (mg) 0.4; Niacin (mg) 20,000; D Cal Pan (mg) 4,400; Co (mg) 120; Cu (mg) 3,200; I (mg) 600; Se (mg) 48; Zn (mg) 24,000; Fe (mg) 16,000; Mn (mg) 40,000; Choline Chloride (mg) 120,000; Antioxidant (mg) 48,000.

Management of Experimental Birds

Two hundred and eighty eight, 1-day-old Isa-brown pullet chicks were used for the experiment. The chicks were brooded altogether for 14 days. After balancing for weight at 15 days of age, the chicks were arranged in a 2 × 4 factorial experimental layout consisting of two groups administered extract of different parts (root or leaves) at different concentration levels (0, 15, 30 and 45 g.l^{-1}). The experiment consisted of 8 treatments replicated three times each with 12 birds per replicate. Prepared aqueous extract of root or leaves of *Petiveria alliacea* at the stated concentration levels were offered to birds in each treatment as drinking water on two consecutive days per week throughout the experimental period. The experiment lasted between 15 to 56 days of age. All treatments receiving aqueous extract of root or leaves *Petiveria alliacea* were free of antibiotic drugs. Experimental birds were raised in a deep litter system with housing temperature ranging between 32 °C (89.6 °F) and 21 °C (69.8 °F) since brooding until the end of the experiment. Birds in all treatments were fed *ad libitum* a formulated starter diet in a mash form (Table 1).

Data Collection

Performance Characteristics

Data on initial body weight, feed intake, water intake, body weight gain, final weight, feed conversion ratio and mortality rate were collected.

$$\text{Daily feed intake (g)} = \text{Feed given (g)} - \text{Feed leftover (g)}$$

$$\text{Daily water intake (ml)} = \text{Volume of water supplied (ml)} - \text{Volume of water left (ml)}$$

$$\text{Average daily feed intake (g)/bird} = \frac{\text{Daily feed intake of the replicate (g)}}{\text{Number of birds in the replicate}}$$

$$\text{Body weight gain (g)} = \text{Final body weight (g)} - \text{Initial body weight (g)}$$

$$\text{Average body weight gain (g)/bird} = = \frac{\text{Final body weight of replicate (g)} - \text{Initial body weight of replicate (g)}}{\text{Number of bird in the replicate}}$$

$$\text{Feed conversion ratio} = \frac{\text{Feed consumed (g)}}{\text{Body weight gain (g)}}$$

$$\text{Mortality (\%)} = \frac{\text{Number of dead birds/Replicate}}{\text{Total number of birds in the replicate}} \times 100$$

Determination of Haematological and Blood Biochemical Indices

Blood Collection

At the end of the study (56 days of age), two millilitres (2 ml) of blood was drawn twice from the brachial vein of two birds in each replicate of all treatments into two different labelled bottles for haematological and serum biochemistry investigations. The blood samples for haematological parameters were collected into bottles pre-treated with ethylene diamine tetraacetate (EDTA), an anticoagulant. Blood samples for biochemical indices was collected into another sample bottle containing no anticoagulant.

Haematological Indices

The haematological indices examined include: Red Blood Cell (RBC), white blood cell (WBC), leucocyte differential count (lymphocytes, monocytes, heterophils, eosinophils, basophils), Packed cell volume (PCV) and haemoglobin (Hb) concentration. RBC and WBC counts were determined using Neubaur chamber method, as described by Lamb (1981). PCV was determined using haematocrit reader according to Benson *et al.* (1989). Haemoglobin was estimated using cyanomethaemoglobin method (Dayyal, 2016).

Serum Biochemical Indices

Serum total protein was determined by the Biuret method (Reinhold, 1953) using a commercial kit (Randox Laboratories Ltd, U.K), while albumin and globulin values were measured by bromocresol green method (Doumas and Biggs, 1971). The serum creatinine and urea nitrogen were estimated by de-proteinisation and Urease-Berhelot colorimetric methods using a commercial kit (Randox Laboratories Ltd, U.K). Free cholesterol was determined using a commercial kit (Quimica Clinica Applicada, S.A).

Intestinal Organ Weight

At the end of the study (56 days of age) two birds with body weights close to the replicate's mean weight were selected from each replicate of all treatments, starved for 12 hours and slaughtered. The gastrointestinal tract was carefully isolated from the carcass. The oesophagus, crop, proventriculus, gizzard, small intestine, large intestine, caecum and liver were separated. Weights of each separated organs were measured using an electronic sensitive weighing scale and expressed as a percentage of live bodyweight.

Table 2. Main effects of aqueous extract of different parts (root and leaf) and concentration of extraction of *Petiveria alliacea* on growth performance of pullet chicks (3 – 8 weeks)

	Plant Part			Concentration of extraction				
Parameters	Root	Leaf	SEM	0 g.l^{-1}	15 g.l^{-1}	30 g.l^{-1}	45 g.l^{-1}	SEM
Initial weight (g/bird)	82.22	82.30	0.04	82.24	82.21	82.28	82.32	0.06
Final weight (g/bird)	412.90	412.74	1.23	410.20	415.47	413.31	412.29	1.57
Total weight gain (g/bird)	330.68	330.43	1.22	327.96	333.26	331.03	329.97	1.54
Weight gain (g/bird/day)	7.87	7.86	0.03	7.81	7.94	7.88	7.86	0.04
Total feed intake (g/bird)	1116.36	1119.46	1.11	1118.35	1117.96	1117.20	1118.14	1.76
Average feed intake (g/bird/day)	26.58	26.65	0.03	26.63	26.62	26.60	26.63	0.04
Feed conversion ratio	3.37	3.39	0.01	3.41	3.35	3.38	3.39	0.02
Total water intake (ml/bird)	3007.62	2870.79	63.72	3150.00^{a}	2942.80^{ab}	2797.50^{b}	2866.40^{b}	73.75
Average daily water intake (ml/bird/day)	71.61	68.35	1.52	75.00^{a}	70.07^{ab}	66.61^{b}	68.25^{b}	1.76
Mortality (%)	0.69	1.39	1.04	2.77	1.38	0.00	0.00	0.79

SEM: Standard error of mean.

$^{a, b}$ Means in the same row not sharing common superscript are significantly ($P < 0.05$) different.

Statistical Analysis

The experiment was arranged in a 2 × 4 factorial experimental layout and data collected were subjected to a completely randomized design. Significant differences among treatment means were determined using a Duncan's Multiple Range Test (Statistical Analysis Software; SAS, 2010).

RESULTS

The main effects of aqueous extract from different parts (root and leaf) and concentration of extraction of *Petiveria alliacea* on growth performance of pullet chicks (3 – 8 weeks of age) are presented in Table 2. Water intake was significantly affected by concentration of extraction. The highest water intake (75.00 ml) was recorded in the control group, while pullets maintained on 30 $g.l^{-1}$ or 45 $g.l^{-1}$ concentration of extraction demonstrated statistically similar and least values of water consumption (66.61 and 68.25 ml, respectively).

The interactive effect of aqueous extract from different parts (root and leaf) and concentration of extraction of *Petiveria alliacea* on growth performance of pullet chicks (3 – 8 weeks of age) is presented in Table 3. Only water intake was significantly ($P < 0.05$) influenced by the interaction of extracts from different parts (root and leaf) and concentration of extraction of *Petiveria alliacea*. The control groups had significantly ($P < 0.05$) higher water intake compared with pullet chicks administered 30 $g.l^{-1}$ concentrations of leaf extract.

The main effects of aqueous extract from different parts (root and leaf) and concentration of extraction of *Petiveria alliacea* on haematological indices and some serum metabolites of pullet chicks at 8 weeks of age are presented in Table 4. Birds offered aqueous root extract had significantly ($P < 0.05$) higher lymphocyte value (67.79 %) compared to birds administered leaf extract (65.75 %). Uric acid was significantly ($P < 0.05$) higher in serum of birds administered leaf extract (4.35 $mg.dl^{-1}$) compared to root extract (3.95 $mg.dl^{-1}$). White blood cell count increased significantly ($P < 0.05$) with increase in concentration of extraction, although only numerical difference was observed between 30 $g.l^{-1}$ and 45 $g.l^{-1}$ concentrations of extraction. Pullets in the control group showed higher ($P < 0.05$) value of heterophil compared to birds offered with other concentrations of extraction. Furthermore, lymphocyte value increased significantly ($P < 0.05$) along with increase

Table 3. Interactive effect of aqueous extract of different parts (root and leaf) and concentration of extraction of *Petiveria alliacea* on growth performance of pullet chicks (3 – 8 weeks)

Plant part	Root				Leaf				
Concentration of extraction	0 $g.l^{-1}$	15 $g.l^{-1}$	30 $g.l^{-1}$	45 $g.l^{-1}$	0 $g.l^{-1}$	15 $g.l^{-1}$	30 $g.l^{-1}$	45 $g.l^{-1}$	SEM
Parameters									
Initial weight (g/bird)	82.19	82.11	82.25	82.31	82.28	82.30	82.30	82.34	0.08
Final weight (g/bird)	409.00	415.55	414.39	412.64	411.39	415.39	412.22	411.94	2.37
Total weight gain (g/bird)	326.81	333.44	332.14	330.33	329.11	333.08	329.92	329.61	2.33
Weight gain (g/bird/day)	7.78	7.94	7.91	7.87	7.84	7.93	7.85	7.85	0.06
Total feed intake (g/bird)	1114.81	1115.25	1116.53	1118.86	1121.89	1120.67	1117.86	1117.42	2.04
Average feed intake (g/bird/day)	26.54	26.55	26.58	26.64	26.71	26.68	26.62	26.61	0.05
Feed conversion ratio	3.41	3.34	3.36	3.38	3.41	3.36	3.39	3.39	0.02
Total water intake (ml/bird)	3132.60^{a}	3094.30^{ab}	2868.10^{ab}	2935.50^{ab}	3167.40^{a}	2791.40^{ab}	2726.90^{b}	2797.30^{ab}	93.86
Average daily water intake (ml/bird/day)	74.59^{a}	73.67^{ab}	68.29^{ab}	69.89^{ab}	75.42^{a}	66.46^{ab}	64.93^{b}	66.60^{ab}	2.23
Mortality (%)	2.77	0.00	0.00	0.00	2.77	2.77	0.00	0.00	1.04

SEM: Standard error of mean.

$^{a, b}$ Means in the same row not sharing common superscript are significantly ($P < 0.05$) different.

Table 4. Main effects of aqueous extract of different parts (root and leaf) and concentration of extraction of *Petiveria alliacea* on haematological indices and some serum metabolites of pullet chicks at 8 weeks of age

	Plant Part			Concentration of extraction				
Parameters	Root	Leaf	SEM	0 g.l^{-1}	15 g.l^{-1}	30 g.l^{-1}	45 g.l^{-1}	SEM
Haematological indices								
Pack Cell Volume (%)	25.06	25.06	0.81	24.68	25.83	24.73	24.98	1.19
Haemoglobin (g.dl^{-1})	8.31	8.42	0.33	8.43	8.60	8.27	8.15	0.49
Red Blood Cell (× 10^{12}.L^{-1})	2.07	2.00	0.08	2.05	2.03	2.02	2.03	0.13
White Blood Cell (× 10^{9}.L^{-1})	11.11	11.05	0.29	9.78^{c}	10.75^{b}	11.87^{a}	11.92^{a}	0.17
Heterophil (%)	29.79	31.17	0.73	33.17^{a}	29.92^{b}	30.50^{b}	28.33^{b}	0.86
Lymphocyte (%)	67.79^{a}	65.75^{b}	0.68	63.67^{c}	67.08^{b}	67.33^{b}	69.00^{a}	0.74
Eosinophil (%)	0.50	0.92	0.17	0.67	0.67	0.67	0.83	0.26
Basophil (%)	0.83	1.08	0.22	1.33	1.17	0.67	0.67	0.30
Monocyte (%)	1.08	1.08	0.26	1.17	1.17	0.83	1.17	0.37
Serum metabolites								
Total protein (g.dl^{-1})	4.56	4.56	0.15	4.37	4.68	4.50	4.68	0.19
Albumin (g.dl^{-1})	2.39	2.41	0.08	2.40	2.47	2.33	2.40	0.11
Globulin (g.dl^{-1})	2.17	2.15	0.12	1.97	2.22	2.17	2.28	0.16
Uric acid (mg.dl^{-1})	3.95^{b}	4.35^{a}	0.18	4.85a	4.25^{b}	4.13^{b}	3.37^{c}	0.16
Creatinine (mg.dl^{-1})	2.46	2.58	0.26	2.48	2.48	2.50	2.62	0.38
Glucose (mg.dl^{-1})	123.12	123.19	3.34	123.73	123.43	122.40	123.07	4.94
Cholesterol (mg.dl^{-1})	153.77	155.22	9.19	200.43^{a}	143.78^{b}	141.93^{b}	131.83^{b}	6.06

SEM: Standard error of mean.
$^{a, b, c}$ Means in the same row not sharing common superscript are significantly ($P < 0.05$) different.

in concentration of extraction, ranging from 63.67 % in control birds to 69.00 % in birds maintained on 45 g.l^{-1} concentration level. The increase in concentration of extraction significantly ($P < 0.05$) decreased uric acid level from 4.85 mg.dl^{-1} in control birds to 3.37 mg.dl^{-1} in birds raised on 45 g.l^{-1} concentration of extraction. Serum cholesterol level was significantly ($P < 0.05$) higher in the control birds compared with other concentration levels.

The interactive effect of aqueous extract of different parts (root and leaf) and concentration of extraction of *Petiveria alliacea* on haematological indices and some serum metabolites of pullet chicks at 8 weeks of age is presented in Table 5. There was significant ($P < 0.05$) increase in white blood cell count as concentration of extraction increased for both groups maintained on root and leaf extracts, although values recorded at 30 g.l^{-1} or 45 g.l^{-1} concentration levels were statistically similar. Values recorded for heterophil varied between 26.67 % in birds administered 45 g.l^{-1} root extract and 34.33 % in the control birds (0 g.l^{-1} root extract). Lymphocyte value was significantly ($P < 0.05$) higher in birds administered root extract compared with values recorded for birds administered leaf extract at each concentration. Moreover, lymphocyte values increased along with increase in concentration of extraction of both plant parts. Uric acid level ranged from 3.07 mg.dl^{-1} in birds administered 45 g.l^{-1} concentration of root extract to 4.90 mg.dl^{-1} in birds offered 0 g.l^{-1} concentration of leaf extract (control). Serum cholesterol was significantly ($P < 0.05$) higher in the control birds compared to birds maintained on other concentrations levels of both root and leaf extract.

The main effects of aqueous extract from different parts (root and leaf) and concentration of extraction of *Petiveria alliacea* on intestinal organ weights of pullet chicks at 8 weeks of age are presented in Table 6. The control birds showed the lowest ($P < 0.05$) caeca weight, while birds maintained on 30 g.l^{-1} or 45 g.l^{-1} concentration of

Table 5. Interactive effect of aqueous extract of different parts (root and leaf) and concentration of extraction of *Petiveria alliacea* on haematological indices and some serum metabolites of pullet chicks at 8 weeks of age

Plant part	Root				Leaf				
Concentration of extraction	0 g.l^{-1}	15 g.l^{-1}	30 g.l^{-1}	45 g.l^{-1}	0 g.l^{-1}	15 g.l^{-1}	30 g.l^{-1}	45 g.l^{-1}	SEM
Parameters									
Haematological indices									
Pack Cell Volume (%)	24.67	26.00	24.67	24.90	24.70	25.67	24.80	25.07	1.87
Haemoglobin (g.dl^{-1})	8.27	8.60	8.13	8.23	8.60	8.60	8.40	8.07	0.75
Red Blood Cell (× 10^{12}.L^{-1})	2.13	2.03	2.03	2.07	1.97	2.03	2.00	2.00	0.19
White Blood Cell (× 10^{9}.L^{-1})	9.83^{c}	10.70^{b}	12.03^{a}	11.87^{a}	9.73^{c}	10.80^{b}	11.70^{a}	11.97^{a}	0.24
Heterophil (%)	34.33^{a}	28.83^{bc}	29.33^{bc}	26.67^{c}	32.00^{ab}	31.00^{ab}	31.67^{ab}	30.00^{bc}	0.97
Lymphocyte (%)	63.00^{e}	68.83^{ab}	69.00^{ab}	70.33^{a}	64.33^{de}	65.33^{d}	65.67^{cd}	67.67^{bc}	0.60
Eosinophil (%)	0.33	0.33	0.33	1.00	1.00	1.00	1.00	0.67	0.31
Basophil (%)	1.33	1.00	0.33	0.67	1.33	1.33	1.00	0.67	0.44
Monocyte (%)	1.00	1.00	1.00	1.33	1.33	1.33	0.67	1.00	0.57
Serum metabolites									
Total protein (g.dl^{-1})	4.20	4.83	4.57	4.63	4.53	4.53	4.43	4.73	0.29
Albumin (g.dl^{-1})	2.30	2.53	2.33	2.40	2.50	2.40	2.33	2.40	0.16
Globumin (g.dl^{-1})	1.90	2.30	2.23	2.23	2.03	2.13	2.10	2.33	0.24
Uric acid (mg.dl^{-1})	4.80^{a}	4.00^{bc}	3.93^{bc}	3.07^{d}	4.90^{a}	4.50^{ab}	4.33^{abc}	3.67^{dc}	0.19
Creatinine (mg.dl^{-1})	2.43	2.37	2.43	2.60	2.53	2.60	2.57	2.63	0.60
Glucose (mg.dl^{-1})	123.33	123.07	122.33	123.77	124.13	123.80	122.47	122.37	7.80
Cholesterol (mg.dl^{-1})	194.87^{a}	148.07^{b}	141.63^{b}	130.50^{b}	206.00^{a}	139.50^{b}	142.23^{b}	133.17^{b}	8.44

SEM: Standard error of mean.

$^{a, b, c, d, e}$ Means in the same row not sharing common superscript are significantly ($P < 0.05$) different.

Table 6. Main effects of aqueous extract of different parts (root and leaf) and concentration of extraction of *Petiveria alliacea* on intestinal organ weights of pullet chicks at 8 weeks of age

	Plant Part			Concentration of extraction				
	Root	Leaf	SEM	0 g.l^{-1}	15 g.l^{-1}	30 g.l^{-1}	45 g.l^{-1}	SEM
Organs (% of body weight)								
Oesophagus	0.41	0.41	0.01	0.40	0.42	0.43	0.41	0.01
Crop	0.96	0.89	0.04	0.92	0.94	0.96	0.88	0.05
Small intestine	5.30	5.57	0.24	5.76	5.59	5.25	5.13	0.31
Large intestine	0.34	0.33	0.01	0.32	0.33	0.34	0.34	0.02
Caeca	0.68	0.69	0.04	0.58^{b}	0.67^{ab}	0.75^{a}	0.75^{a}	0.04
Proventriculus	0.69	0.72	0.03	0.70	0.72	0.70	0.71	0.05
Empty gizzard	2.31	2.22	0.05	2.32	2.20	2.24	2.29	0.07
Liver	2.05	2.01	0.04	2.02	2.00	2.03	2.07	0.06

SEM: Standard error of mean.

$^{a, b}$ Means in the same row not sharing common superscript are significantly ($P < 0.05$) different.

Table 7. Interactive effect of aqueous extract of different parts (root and leaf) and concentration of extraction of *Petiveria alliacea* on intestinal organ weights of pullet chicks at 8 weeks of age

Plant part	Root				Leaf				
Concentration of extraction	0 g.l^{-1}	15 g.l^{-1}	30 g.l^{-1}	45 g.l^{-1}	0 g.l^{-1}	15 g.l^{-1}	30 g.l^{-1}	45 g.l^{-1}	SEM
Organs (% of body weight)									
Oesophagus	0.39	0.42	0.43	0.41	0.41	0.41	0.42	0.40	0.01
Crop	0.93	1.03	1.02	0.88	0.91	0.86	0.91	0.88	0.08
Small intestine	5.51	5.28	5.12	5.30	6.02	5.90	5.39	4.97	0.41
Large intestine	0.32	0.35	0.35	0.32	0.32	0.31	0.34	0.35	0.02
Caeca	0.59	0.65	0.72	0.79	0.58	0.70	0.78	0.72	0.05
Proventriculus	0.73	0.66	0.72	0.65	0.66	0.78	0.68	0.78	0.06
Empty gizzard	2.35	2.16	2.31	2.42	2.29	2.25	2.17	2.16	0.10
Liver	2.05	2.04	2.02	2.09	1.99	1.97	2.04	2.05	0.10

SEM: Standard error of mean.

extraction had similar and highest caeca weights.

The interactive effect of aqueous extract from different parts (root and leaf) and concentration of extraction of *Petiveria alliacea* on intestinal organ weights of pullet chicks at 8 weeks of age are presented in Table 7. The interaction had no significant ($P > 0.05$) influence on all intestinal organ weights examined.

DISCUSSION

The similarities in growth performance observed in this study indicated that aqueous extract from root and leaf of *Petiveria alliacea* offered to pullet chicks constitutes main important phytochemicals. Phytochemicals have been reported as bioactive non-nutritive compounds (Liu, 2013) and, therefore, may have no substantial contribution to growth performance. There is also suggestion that aqueous extract from root and leaf of *Petiveria alliacea* at concentration levels up to 45 g.l^{-1} contains minimal level of anti-nutritional phytochemicals, since there was no detrimental effect on growth of pullet chick. Similarly, Odetola *et al.* (2019) reported no significant difference in all growth parameters examined when broiler birds were fed diets supplemented with graded levels of *Petiveria alliacea* root meal at 500 g, 1000 g, 1500 g, 2000 g and 2500 g.100 kg^{-1} of feed. However, Muhammad *et al.*, (2018) found improvement in growth performance of growing pullets fed diet containing 1000 mg of *Petiveria alliacea* root meal per kilogram of feed. Disparity in these results may be attributed to the stage of growth of the pullet chicks and mode of application of *Petiveria alliacea.*

The reduced water consumption observed in pullet chicks maintained on the extracts from root and leaf of *Petiveria alliacea* might not be a factor of taste, since chickens have been reported to have few taste receptors with sweet taste receptor missing and bitter taste receptor very few (Berkhoudt, 1985; Roura *et al.*, 2013). Odour and appearance were stated as important factors in determining what birds consume, like other mono-gastric animals (Baldwin, 1976; Mellor, 2000). Hence, the lowered water intake can be credited to water discolouration and characteristic strong odour of *Petiveria alliacea* resulting from the presence of sulfurate compounds (De Sousa *et al.*, 1990).

Similarities in PCV, Hb and RBC of birds in all treatments signified that administration of aqueous extract of *Petiveria alliacea* did not trigger anaemic or polycythaemia conditions in birds. Therefore, it supported normal physiological functions of the body. All values recorded in this study fell within the range ($2.0 - 3.5 \times 10^{12}$.L^{-1}, 6.5 – 13 g.dl^{-1} and 22 – 43 % for RBC, Hb and PCV respectively) earlier reported by Santoso *et al.* (2017).

The increase in white blood cell count and lymphocyte differential can be considered as a result of enhancement in the immune system instigated

by the experimental plant. This boost ability of the birds to fight against foreign bodies, thereby, strengthening the resistance to diseases (Alegre and Clavo, 2007). *Petiveria alliacea* was reported to possess immune-stimulating properties due to the presence of dibenzyl trisulfide (Quadros *et al.*, 1999; Rosner *et al.* 2001). Randle *et al.* (2018) also stated that water extract of *Petiveria alliacea* stimulated lymphocyte, interferon and interleukin production. This result is synonymous to the findings of Sobayo *et al.* (2018), who reported an increase in the lymphocyte count of broiler birds fed 100 ppm or 500 ppm dietary inclusion of *Petiveria alliacea* root and leaf meal. Higher lymphocyte differential observed in pullet chicks administered root extract indicated that root extract tends to promote production of antibodies and cytotoxic cells compared to leaf extract. Values recorded for white blood cell count fell within the range ($5.0 – 15.00 \times 10^9.L^{-1}$) reported by McDonald (1996).

The concentration dependent reduction in serum uric acid level caused by the administration of aqueous extract of root and leaf of *Petiveria alliacea* in this study suggested that the extracts promoted protein utilization, owing to the fact that high serum uric acid level signals poor dietary protein utilization (Oduguwa and Ogunmodede, 1995; Awosanya *et al.*, 1999). This also indicated improved renal functionality (Oni *et al.*, 2018). This corresponds to the finding of Sobayo *et al.* (2018), who observed low serum uric acid concentration in broilers fed 500 or 1500 ppm dietary inclusion of *Petiveria alliacea*.

The observed decrease in serum cholesterol level may suggest inhibition of fatty acid synthesis by *Petiveria alliacea*. The organosulfur compounds present in *Petiveria alliacea*, similar to those in garlic and onion (Randle *et al.*, 2018), are responsible for its characteristic garlic odour. These compounds have been found to inhibit squalene epoxidase, which is involved in the synthetic pathway of cholesterol (Khan *et al.*, 2007). Elson and Qureshi (1995) also stated that extracts from plants may lower blood cholesterol in chickens by inhibiting 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the enzyme involved in cholesterol synthesis. This result is similar to the finding of Stanacev *et al.* (2011), who reported hypocholesterolaemic effect in blood serum of garlic-treated broilers.

Also, Oleforuh-Okoleh *et al.* (2015) opined that serum cholesterol was reduced when broiler chickens were administered aqueous extract of ginger and garlic. However, Odetola *et al.* (2019) reported an increase in serum cholesterol of broiler chickens fed 2500 g of *Petiveria alliacea* root meal per 100 kg of feed. This discrepancy may arise from variations in preparation methods, which may affect the stability of active organo-sulphur compounds (Poojary *et al.*, 2017). Similarities in weight of oesophagus, crop, gizzard, proventriculus, small intestine, large intestine and liver indicated that the administration of aqueous extract from root and leaves of *Petiveria alliacea* at all tested concentrations did not interfere with normal development of these organs. Improvement in the caeca weight, recorded in this study, could be a result of physiological adjustment to increased biological activities within the caeca. The caeca has been found to be a site for fermentation and further digestion of feed, for utilization and absorption of water and nitrogenous components, for microbial action and as a site for production of immunoglobulins (Clench and Mathias, 1995). Therefore, the antimicrobial (Kim *et al.*, 2006; Ekunseitan *et al.*, 2016) and immunomodulatory (Williams *et al.*, 2007; Santander *et al.*, 2012) activities of *Petiveria alliacea* might have enchanced biological processes within the caeca and, thus, the consequent adjustment in weight. Similarly, Rougiere and Carre (2010) reported a rise in the activity within the caeca and subsequent increase in the caeca weight by inclusion of 15 % sunflower hulls into the diet of broiler chickens.

CONCLUSION

It is concluded that aqueous root extract of *Petiveria alliacea* at 45 $g.l^{-1}$ concentration level best enhanced immune cell proliferation and reduced serum uric acid and cholesterol content in pullet chicks without compromising their growth.

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BEHAVIOURAL AND PHYSIOLOGICAL RESPONCES OF BALADI RED AND NEW ZEALAND WHITE RABBIT TO NATURAL OESTRUS INDUCTION METHODS

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ABSTRACT

This experiment was designed to study the behavioural and physiological responses of two breeds of rabbits to natural oestrus induction methods in non-receptive female rabbits to mating. One hundred multiparous female rabbits from Baladi Red (BR) and New Zealand White (NZW) breeds (50 from each breed) and twenty mature bucks from two breeds (10 from each breed) were used in the study. The following two methods were used: (1) doe-litter separation in the suckling females, or (2) presence of a doe beside buck cage in the non-suckling females. Basic behavioural (time of standing, walking and sitting %) and sexual behavioural activities (frequency of male circling around female, female circling around male, male mounting female and actual mating) were recorded for each male and female rabbits. Receptivity and conception rates were calculated in each treatment group. Also, serum concentrations of estradiol-17β hormone were determined in does under investigation. The results of this experiment indicated that animals after application of natural inducing oestrus treatments are more active than before treatments. Time of standing was significantly higher than time of sitting in both breeds after treatments compared with those before treatments. Moreover, animals after application of treatments showed significantly higher frequency of female circling around male, male mounting female and actual mating, and insignificantly higher frequency of male circling around female, than those values recorded in animals before application of treatments. Oestrogen levels significantly increased after presence doe beside buck cage and insignificantly increased after doe-litter separation in both breeds. Both treatments showed pronounced improvement in terms of receptivity and conception rates, irrespective of breed. NZW does were significantly superior over BR does in most studied traits. Highly significant positive correlations were found between both sexual behaviour and oestrogen level with receptivity and conception rates. Generally, natural methods used to induce oestrus led to a positive change in the basic and sexual behaviour as well as improvement in the physiological performance of non-receptive female rabbits for mating.

Key words: oestrus induction; behaviour; doe-litter separation; male effect; rabbit does

INTRODUCTION

Recently, bio-stimulation methods to synchronize estrus in rabbits does were used to induce receptivity rather than hormonal treatments. The natural methods used such as female beside male cage (Enas Abd El Wahed, 2017), feed restriction (Mehaisen and Abbas, 2014), mother-litter separation (Ilès *et al.*, 2013), flushing and changes of female cages before mating (Manal, 2010) or lighting regime (McNitt, 1992).

All these studies showed a significant improvement in the physiological, reproductive and productive performance of rabbit does. On the other hand, mammalian reproduction starts with mating behavior and terminates when the young are weaned. Between these two events, there are complex chain of behavioural phenomena, which are critical for the survival of the youngs (Marai and Rashwan, 2003). Rabbits are induced ovulators with no regular oestrous cycle (Maertens *et al.*, 1995).

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The stimulus of mating initiates the ovulation process, due to the surge of GnRH from hypothalamus after mating by physical stimulation of genital areas causing the LH peak, which initiates the ovulatory process (McNitt, 1992). In nursing rabbits, sexual receptivity and fertility is depressed during the period of lactation, presumably through a hormonal antagonism between prolactin and gonadotropin release. These endocrine changes would explain the activation of ovarian function (Gonzalez-Mariscal, 2001).

Nevertheless, there are few studies on the behavioural changes of female rabbits during accepted and refused matting. Therefore, the objectives of the present study were to record and investigate the behavioural responses of two breed of rabbits under two natural methods to induce oestrus in non-receptive doe rabbits. In addition, determination of some reproductive parameters was done during the study.

MATERIAL AND METHODS

This experiment was carried out at a Rabbitry of the Experimental Farm, Faculty of Agriculture, Suez Canal University, Ismailia, Egypt. Two bio-stimulation methods were used to induce oestrus in normally healthy and non-receptive rabbit females. These methods were: 1) doe-litter separation in suckling females (n = 61) for 24 h, and 2) presence of doe beside male cages in non-suckling females (n = 39) for 24 h. Each method was chosen according to the female status (suckling or non-suckling).

Animals, husbandry and experimental design

One hundred multiparous female rabbits from Baladi Red (BR) and New Zealand White (NZW) breeds (50 from each breed) and twenty mature bucks form two breeds (10 from each breed) were used in the study. Animals were free of any external parasites or skin diseases and were not treated hormonally before this experiment. Age of animals ranged from 10–12 months.

Animals were housed individually in galvanized wired cages (40x40x30 cm), where feed and water were provided *ad libitum*. Animals were fed on basal pellet ration contained yellow corn, soybean meal, corn gluten, minerals and vitamins premix, bone and molasses. The calculated chemical components of the diet were: 17 % crude protein, 2.8 % fat, 10 % crude fibre and 2600 kcal digestible energy/kg diet. Lighting system was 16 h light/8 h dark in the rabbitry during the experimental period. Does were transferred to the rabbit buck cages for natural mating process and kept under examination until natural mating was successfully completed. The experiment lasted seven months, from September to April. At the start of the experiment, all females were non-pregnant. All females were naturally mated. Bio-stimulation methods were applied on females, who rejected mating once at any time during the experiment.

All procedures were reviewed and approved by the Faculty of Agriculture, Suez Canal University and complied with the Institutional Animal Care and Use Committee of Suez Canal University.

Studied traits

Behavioural activities

Basic and sexual behavioural activities of males and females of the two breeds were studied during the experimental period. The behavioural traits of animals were recorded after and before application of treatments in both breeds by using a video camera for one hour during the morning from 9 to12 am. Each doe in each treatment was transferred into a buck cage of the same breed. From the video tapes, the basic behavioural activities (percentage of bucks and does standing, sitting or walking) were recorded at 3 min interval (sampling time) and scored according to Khalil *et al.* (2014). In addition, sexual behavioural patterns of both sexes, such as frequency of male circling around female, female circling around male, male mounting female and actual mating (Hassan *et al.*, 2015) were recorded during sexual desire and mating.

Receptivity and conception rates

Receptivity rate was determined in rabbit doe breeds after application of the selected bio-stimulation method in suckling or non-suckling females. Receptivity was determined by the willingness of the doe to mate combined with signs of oestrus, such as swelling of the vulva, vulva colour, exposition of the rear quarters and lordosis. Doe rabbits were transferred to male cages to check receptivity for 5 min and, if refused, the treatment was repeated again on the next day.

Litter separation method was applied on the next day after delivery. Conception rate was diagnosed in rabbit does after 12 – 15 days of successful mating. Abdomen palpation was applied on assumed pregnant does by the same technician. Data on reception and conception rates were calculated as a percentage from non-responsive does.

Blood sampling and oestrogen hormone determination

Blood samples were collected from the ear vein of females, which rejected mating; after applying the bio-stimulation method from females that accepted mating, samples were taken after one hour following treatment. The blood was left for coagulation at the fridge temperature (at 4 °C) before centrifugation at 4000 rpm for 20 min to separate serum. Serum samples were frozen at -20 °C until hormonal analyses. Oestrogen hormone was determined by using VIDAS Estradiol II kits from bioMerieux SA, France. The lower detection limit was 9 pg.mL^{-1}, the intra- and inter-assay CV were 7.5 % and 9.5 %, resp.

Statistical Analyses

Data were analysed by using the General Linear Model (GLM) procedure of SAS (SAS Institute Inc., 2004). Differences (LSD) between means were tested using a Duncan's multiple range test (Duncan, 1955). Correlation coefficients among traits were estimated.

Behavioural activities and hormonal profile

Factorial design was applied for the evaluation of the experiment. Three-way analysis of variance (ANOVA-test) with three-way interactions was carried out using the following model:

$Y_{ijkl} = \mu + B_i + D_j + T_k + B*D_{ij} + B*T_{ik} + D*T_{jk} + B*D*T_{ijk} + e_{ijkl}$,

where:

Y_{ijkl} = the observation on the kth individual from the ith breed in jth doe status.

μ = the overall mean.

B_i = the fixed effect of the ith breed (i = Baladi and NZW).

D_j = the fixed effect of the jth doe status (presence beside male and litter separation).

T_k = the fixed effect of the kth time (before and after treatment).

$B*D_{ij}$ = the interaction among ith breed and jth doe status.

$B*T_{ik}$ = the interaction among ith breed and kth time.

$D*T_{ik}$ = the interaction among jth doe status and kth time.

$B*D*T_{ijk}$ = the interaction among ith breed, jth doe status and kth time.

e_{ijkl} = the random error associated with the individual ijkl.

Receptivity and conception rates

Factorial design was applied for the evaluation of the experiment. Two-way analysis of variance (ANOVA-test) with two-way interactions was carried out using the following model:

$Y_{ijk} = \mu + B_i + D_j + B*D_{ij} + e_{ijk}$

Where:

Y_{ijk} = the observation on the kth individual from the ith breed in jth doe status.

μ = the overall mean.

B_i = the fixed effect of the ith breed (i = Baladi and NZW).

D_j = the fixed effect of the jth doe status (presence beside male and litter separation).

$B*D_{ij}$ = the interaction among ith Breed and jth Doe status.

E_{ijk} = the random error associated with the individual ijk.

RESULTS

Basic behavioural activities

Data on basic behavioural activities of BR and NZW rabbit breeds are presented in Tables 1 and 2. Significant differences ($P \leq 0.05$) in standing and walking behavioural activities between two breeds were found. NZW does had significantly ($P \leq 0.05$) lower time of standing (28.17 %) and higher ($P \leq 0.05$) time of walking (23.41 %) than those recorded in BR does (35.71 and 16.66 %, respectively). However, no significant differences were found between two treatments in basic behavioural activities. Moreover, after application of natural inducing oestrus treatments on rabbit does, which refused mating, standing behavioural activity was significantly ($P \leq 0.05$) increased but sitting behavioural activity was decreased ($P \leq 0.01$), compared with those before treatments in both breeds (Table 1).

In addition, significant ($P \leq 0.01$) differences were found among the treatment effect interactions in all basic behavioural activities. The highest ($P \leq 0.01$) standing behaviour was recorded in BR does treated with beside males (47.61 %), but the lowest ($P \leq 0.01$) value was recorded in NZW does before treated with doe-litter separation (17.46 %). However, the lowest time of walking behaviour was observed in BR does after treated with beside males (9.52 %),

Table 1. Basic behavioural activities (%) in NZW and BR rabbit does after inducing oestrus to females (mean ± SE)

		Standing (%)	Walking (%)	Sitting (%)
Breed	NZW	28.17 ± 2.37^{b}	23.41 ± 1.48^{a}	48.41 ± 2.97
	BR	35.71 ± 2.58^{a}	16.66 ± 2.23^{b}	47.61 ± 1.85
	P-value	0.043	0.020	0.823
Type of treatment	Doe-Litter Separation (DLS)	28.57 ± 2.55	20.23 ± 1.95	51.19 ± 2.87
	Doe Beside Male (DBM)	35.31 ± 2.51	19.84 ± 2.33	44.84 ± 1.48
	P-value	0.07	0.897	0.063
Time	Before (control)	28.17 ± 2.44^{b}	19.44 ± 1.80	52.38 ± 2.48^{a}
	After (treated)	35.71 ± 2.52^{a}	20.63 ± 2.44	43.65 ± 1.64^{b}
	P-value	0.043	0.699	0.008
Interactions				
Breed*Treatment*Time	NZW*DLS*Before	17.46 ± 1.58^{c}	19.04 ± 2.74^{abc}	63.49 ± 3.17^{a}
	NZW*DLS*After	30.15 ± 4.19^{b}	28.57 ± 2.74^{a}	41.26 ± 1.58^{b}
	NZW*DBM*Before	33.15 ± 2.74^{b}	20.63 ± 1.58^{ab}	46.03 ± 4.19^{b}
	NZW*DBM*After	31.74 ± 4.19^{b}	25.39 ± 1.58^{a}	42.85 ± 2.74^{b}
	BR*DLS*Before	33.33 ± 5.49^{b}	14.28 ± 2.74^{bc}	52.38 ± 2.74^{b}
	BR*DLS*After	33.33 ± 2.74^{b}	19.04 ± 2.74^{abc}	47.61 ± 5.49^{b}
	BR*DBM*Before	28.58 ± 2.74^{b}	23.80 ± 5.49^{ab}	47.61 ± 2.74^{b}
	BR*DBM*After	47.61 ± 0.0^{a}	9.52 ± 2.74^{c}	42.85 ± 2.74^{b}
P-value		0.001	0.009	0.006

^{a,b,c} Means within column not sharing a common superscript differed significantly.

but the highest value was recorded in NZW does after treated with doe-litter separation (28.57 %). NZW does before treated with doe-litter separation had significantly ($P \leq 0.01$) increased time of sitting behaviour compared to all treated groups.

In relation to males, no significant differences were found between bio-stimulation methods and breeds in basic behaviour activities, but significant ($P \leq 0.05$) differences were found in their interactions in time of standing and sitting behaviour activities. The highest ($P \leq 0.05$) standing behaviour was recorded in BR breed before treated with beside males (66.66 %), but the lowest value was observed in BR after treated with doe-litter separation (49.20 %). Moreover, sitting behaviour was significantly ($P \leq 0.01$) increased in bucks of BR breed after treated with doe-litter separation compared to other treatment groups.

Sexual behavioural activities

Frequency of male circling around female (MCAF), female circling around male (FCAM), male mounting female (MMF) and actual mating (AM), as affected by treatments and their interactions, are presented in Table 3. The results show that NZW breed had significantly ($P \leq 0.05$) increased frequency of AM, but insignificantly higher frequency of MCAF, FCAM and MMF, than those values recorded in the BR breed.

Significant ($P \leq 0.01$) differences in all sexual behaviour traits, except AM, were found due to the oestrus bio-stimulation methods. The group treated with beside males had significantly ($P \leq 0.01$) higher frequency of MCAF, FCAM and insignificantly higher frequency of AM, but significantly lower frequency of MMF than those recorded in the group treated with doe-litter separation.

Analysis of variance showed significant ($P \leq 0.01$) differences between the control and

Table 2. Basic behavioural activities (%) in NZW and BR rabbit bucks after inducing oestrus to females (mean ± SE)

		Standing (%)	Walking (%)	Sitting (%)
Breed	NZW	59.12 ± 1.48	25 ± 0.85	15.87 ± 1.22
	BR	59.12 ± 2.89	22.22 ± 1.07	18.65 ± 2.58
	P-value	0.999	0.055	0.342
Type of treatment	Doe-Litter Separation (DLS)	56.74 ± 2.15	22.61 ± 1.03	20.63 ± 2.14^{a}
	Doe Beside Male (DBM)	61.50 ± 2.22	24.60 ± 0.98	13.88 ± 1.36^{b}
	P-value	0.138	0.179	0.014
Time	Before (control)	60.31 ± 1.88	23.41 ± 1.09	16.26 ± 1.48
	After (treated)	57.93 ± 2.61	23.80 ± 1.01	18.25 ± 2.47
	P-value	0.467	0.792	0.499
Interactions				
Breed*Treatment*Time	NZW*DLS*Before	58.73 ± 1.58^{ab}	22.22 ± 1.58	19.04 ± 2.74^{b}
	NZW*DLS*After	60.31 ± 4.19^{ab}	25.39 ± 1.58	14.28 ± 2.74^{b}
	NZW*DBM*Before	57.14 ± 2.74^{ab}	26.98 ± 1.58	15.87 ± 1.58^{b}
	NZW*DBM*After	60.31 ± 4.19^{ab}	25.39 ± 1.58	14.28 ± 2.74^{b}
	BR*DLS*Before	58.73 ± 3.17^{ab}	22.22 ± 3.17	19.04 ± 0.00^{b}
	BR*DLS*After	49.20 ± 5.72^{b}	20.63 ± 1.58	30.15 ± 4.19^{a}
	BR*DBM*Before	66.66 ± 5.49^{a}	22.22 ± 1.58	11.11 ± 4.19^{b}
	BR*DBM*After	61.90 ± 5.49^{ab}	23.80 ± 2.74	14.28 ± 2.74^{b}
P-value		0.050	0.392	0.010

^{a,b,c} Means within column not sharing a common superscript differed significantly.

treated animals in all sexual behaviour traits except MCAF. Treated animals after application of treatments had significantly ($P \leq 0.01$) higher frequency of FCAM, MMF and AM, and insignificantly ($P = 0.06$) higher frequency of MCAF, than those values recorded in the control animals before application of treatments.

Significant ($P \leq 0.01$) differences were found among the treatment effect interactions in all basic behavioural activities. The highest frequencies of MAF and FAM were recorded in BR does treated with beside males, but the lowest values were recorded in BR does before treated with doe-litter separation. The lowest frequency of MMF was observed in BR does before treated with beside males, but the highest value was recorded in NZW does after treated with doe-litter separation. Moreover, AM frequency was significantly increased ($P \leq 0.01$) in all treated groups compared to the control animals before application of treatments.

Oestrogen hormonal profile

Table 3 shows levels of estradiol-17β (E_2) in NZW and BR breeds before and after application of treatments. No significant differences were found between both breeds in E_2 levels. However, NZW breeds showed slightly higher E_2 level than that in BR breed. Overall effects of natural inducing oestrus methods showed significant ($P \leq 0.01$) differences in E_2 level. Females treated with presence beside male had significantly ($P \leq 0.01$) higher E_2 (53.4 pg.mL^{-1}) than those measured in females treated with doe-litter separation method (47.5 pg.mL^{-1}). The overall effect of time (before and after treatment) on E_2 levels was significant ($P \leq 0.00$). Concentration of E_2 after application of treatments was significantly ($P \leq 0.01$) higher (54.3 pg.mL^{-1}) than those recorded before application of the treatment (46.6 pg.mL^{-1}). The interaction effects among treatments showed significant ($P \leq 0.01$) differences in serum E_2 level. The highest ($P \leq 0.01$) E_2 level was recorded in NZW

Table 3. Frequency (number of movements per hour) of some sexual behavioural activities traits of NZW and BR rabbits and estradiol-17β (E_2) as affected by treatments (mean ± SE)

Breed	Male circling around female (No)	Female circling around male (No)	Male mounting female (No)	Actual mating (No)	E_2 (pg.mL^{-1})
NZW	111.7 ± 9.2	105.5 ± 10.8	53.1 ± 3.3^{a}	4.7 ± 1.44	51.0 ± 1.9
BR	95.2 ± 15.2	86.0 ± 16.8	44.0 ± 1.9^{b}	4.2 ± 1.33	49.8 ± 1.1
P-value	0.365	0.340	0.028	0.802	0.601
Type of treatment					
Doe-Litter Separation (DLS)	68.7 ± 8.2^{b}	70.0 ± 10.9^{b}	54 ± 3.1^{a}	4.2 ± 1.33	47.5 ± 0.7^{b}
Doe Beside Male (DBM)	138.2 ± 6.1^{a}	121.8 ± 13.2^{a}	43.1 ± 1.9^{b}	4.7 ± 1.44	53.4 ± 1.8^{a}
P-value	0.000	0.000	0.008	0.802	0.006
Time					
Before (control)	87.2 ± 9.6	60.9 ± 7.8^{b}	43.2 ± 2.2^{b}	0.00^{b}	46.6 ± 0.6^{b}
After (treated)	119.5 ± 13.7	130.5 ± 11.7^{a}	53.9 ± 2.9^{a}	4.5 ± 0.4^{a}	54.3 ± 1.6^{a}
P-value	0.066	0.000	0.009	0.000	0.000
Interactions					
Breed*Treatment*Time					
NZW*DLS*Before	69.0 ± 1.1^{e}	70.0 ± 1.1^{e}	55 .0 ± 1.7^{b}	0.0^{c}	45.6 ± 1.2^{c}
NZW*DLS*After	113.0 ± 1.3^{d}	120.0 ± 2.8^{c}	70.0 ± 1.1^{a}	4.8 ± 0.3^{a}	48.5 ± 1.7^{c}
NZW*DBM*Before	110.0 ± 2.8^{d}	74.6 ± 1.4^{e}	40 .0 ± 1.1^{e}	0.0^{c}	47.0 ± 1.7^{c}
NZW*DBM*After	155.0 ± 2.8^{b}	157.7 ± 1.5^{b}	40.0 ± 1.4^{cd}	4.5 ± 0.4^{a}	62.7 ± 2.6^{a}
BR*DLS*Before	45.0 ± 1.1^{f}	18.0 ± 1.1^{f}	43 .0 ± 1.1^{de}	0.0^{c}	46.3 ± 1.2^{c}
BR*DLS*After	48.0 ± 1.1^{f}	72.3 ± 1.4^{e}	48.0 ± 1.1^{c}	3.5 ± 0.5^{b}	49.7 ± 1.5^{c}
BR*DBM*Before	125.0 ± 2.8^{c}	81.0 ± 1.1^{d}	35.0 ± 1.1^{f}	0.0^{c}	47.2 ± 1.7^{c}
BR*DBM*After	163.0 ± 1.3^{a}	173.0 ± 1.3^{a}	50.0 ± 2.8^{c}	4.8 ± 0.3^{a}	56.2 ± 1.1^{b}
P-value	0.000	0.000	0.000	0.000	0.000

^{a,b,c,d,e,f} Means within column not sharing a common superscript differed significantly.

does after treated with beside males, but the lowest values were recorded in NZW does before treated with doe-litter separation. These results indicated different mode of action of both treatments on the inducing oestrus in BR and NZW breeds.

Receptivity and conception rates

Table 4 shows receptivity and conception rates in NZW and BR does, as affected by treatments. Bio-stimulation methods significantly ($P \leq 0.05$) increased receptivity percentage in females that rejected mating, in both breeds, compared with the control animals. This increment was 15 % in both breeds (Table 3). However, females treated with doe-litter separation had insignificantly ($P = 0.14$) increased receptivity percentage (6.26 %) compared with females treated with beside males, irrespective of breeds. On the other hand, the interaction effects of treatment and rabbit breeds showed insignificant ($P = 0.31$) differences between treatments within the breeds (Table 4). A significant difference was found between two bio-stimulation methods in the conception rate. Females treated with beside females had significantly higher ($P \leq 0.00$) conception rate (72.29 %) than those in females treated with doe-litter separation (55.21 %). Moreover, no significant differences were detected between the breeds ($P = 0.74$) and the interaction effects ($P = 0.06$) in the conception rate (Table 4). NZW does had insignificantly higher conception rate

Table 4. Receptivity and conception rates (%) in NZW and BR rabbits after inducing oestrus (mean ± SE)

		Receptivity %	Conception rate
Before treatment (Control group)			
Breed	NZW	54.80 ± 3.54	69.36 ± 5.81
	BR	60.83 ± 5.15	62.68 ± 4.94
	Overall	57.81 ± 3.26^{B}	65.56 ± 4.45
	P-value	0.354	0.425
After treatment (Treated group)			
Breed	NZW	70.04 ±3.13	65.72 ±5.01
	BR	75.20 ± 2.86	63.60 ± 4.13
	Overall	72.84 ± 3.86^{A}	64.48 ± 4.18
	P-value	0.227	0.743
Treatment	Doe-Litter Separation (DLS)	76.32 ± 3.13	55.21 ± 4.98^{b}
	Doe Beside Male (DBM)	70.06 ± 2.85	72.29 ± 3.95^{a}
	P-value	0.142	0.007
Interactions	NZW*DLS	72.79 ± 4.93	55.45 ± 8.04
	NZW*DBM	67.95 ± 4.07	73.55 ± 6.14
	BR*DLS	78.95 ± 4.06	55.03 ± 6.36
	BR*DBM	71.89 ± 4.00	71.20 ± 5.17
	P-value	0.314	0.065

^{a,b} Means within column not sharing a common superscript differed significantly.
$^{A, B}$ Means within column not sharing a common superscript differed significantly.

(65.72 %) than those recorded in BR does (63.60 %). Also, the highest conception rate was obtained in NZW does treated with beside males (73.55 %) than those recorded in BR does (55.03 %).

Correlation coefficient among some studied traits

Results in Table 5 demonstrate prevalent moderate-to-high significant positive correlation between both E_2 levels with each of some basic and sexual behavioural activities, such as standing females, walking males, males around females, females around males and actual mating. In addition, positive correlation was observed between actual mating with each of male circling around females, females circling around male, male mounting females, receptivity and conception rates. Also, moderate-to-high positive correlation was determined between receptivity and standing females, male around females, actual mating and E_2. On the other hand, low-to-moderate negative correlation was observed between E_2 levels and both sitting males and females, and with males mounting females.

DISCUSSION

Results of the present study showed clearly that both bio-stimulation methods significantly affected and markedly improved most behavioural activities and physiological traits. After treatments, animals had significantly decreased time of sitting (flattening on the floor cage) and increased the time of standing, while insignificantly increased the time of walking behavioural activities compared with those recorded before treatments. Moreover, frequency of female circling around male, male mounting female and actual mating after application of natural inducing oestrus was also increased compared with before treatments.

Preparation of a receptive doe for mating takes few seconds; the sexually mature doe in heat accepts the male after 2 or 3 initial attempts. The libido in the male is so vigorous that he becomes tired if 4 or 5 attempts to mate failed. A successful mating is recorded from the characteristic wooing sound of the male followed by his falling off the female either in a backward or in a sideway direction

Table 5. Correlation coefficient among some behavioural traits and oestrogen hormone

	Wlk F	Sit F	St M	Wlk M	Sit M	Male circling around female	Female circling around male	Male mount. Female	Actual mate.	E_2	Rec.	Con. Rate
St F	-0.513	-0.587	-0.095	-0.016	-0.072	0.457	0.429	-0.261	0.443	0.492	0.476	0.723
Wlk F	1	-0.234	0.122	0.499	-0.257	-0.024	0.101	0.289	0.124	0.031	0.181	0.141
Sit F		1	-0.135	-0.519	0.297	-0.597	-0.678	-0.008	-0.653	-0.643	-0.567	-0.969
St M			1	0.331	-0.932	0.299	0.303	-0.247	-0.127	0.136	-0.398	0.154
Wlk M				1	-0.637	0.668	0.478	0.075	0.195	0.407	-0.211	0.548
Sit M					1	-0.489	-0.408	0.186	0.059	-0.234	0.412	-0.349
Male circling around female						1	0.830	0.101	0.581	0.747	0.030	0.662
Female circling around male							1	0.227	0.808	0.857	0.181	0.731
Male mount. Female								1	0.524	-0.044	0.482	0.114
Actual mate.									1	0.743	0.683	0.740
E_2										1	0.561	0.601
Rec.											1	0.496
Con. Rate												1

Wlk F = Walking females, Sit F = Sitting females, St M = Standing males, Wlk M = Walking Males, Sit M = Sitting males, E_2 = Estradiol-17β (pg.mL^{-1}), Rec. = Receptivity (%), Con. Rate = Conception rate (%).

(Lebas *et al.*, 1986; Pfaus *et al.* (2015). In contrast, a doe in low receptivity refuses to accept the buck. She runs or keeps her head up in the air, she flattening on the floor of the cage, circling, circling and flattening, running away quickly from the male, trying to mount the male and occasionally showed an aggressive behaviour (Forcada and Abecia, 1990; McCroskey, 2000).

In this context, serum concentration of E_2 increased significantly after application of natural inducing oestrus compared with before treatments. Also, receptivity percentage and conception rate improved after treatments by 72.84 and 64.48 %, respectively. Moreover, highly positive correlations were found between E_2 concentration and both sexual and basic behavioural activity, such as frequency of female circling around male, male circling around female, actual mating and time of female standing behaviour (r = 0.857, 0.747, 0.743 and 0.492 respectively). These results confirmed that, oestrogen hormone is very responsible for display doe sexual behaviour and acceptance of the male (Ilès *et al.*, 2013). Several studies have demonstrated that separation of the doe from its litter for short periods of time is very effective in stimulating ovarian activity of the doe. This, in turn, leads to raise the level of oestrogen in the blood (Manal, 2010; Ilès *et al.*, 2013).

Our results are similar with the results obtained by Virag *et al.* (1999), who found that using doe-litter separation method for one day or more on day 9 after suckling increased fertility by 20 %. Also, Rebollar *et al.* (2008) and Ilès *et al.* (2013) recorded that using doe-litter separation method for 48 h improved sexual receptivity and, consequently, fertility of the lactating does. However, McNitt (1992) and Rebollar *et al.* (1992) attributed the reduction of receptivity percentage and failure of mating during suckling period to the rise in prolactin and low secretion of oestrogen by the ovaries, which has a negative effect on receptivity percentage and sexual behaviour. Also,

these results are nearly consistent with Rebollar *et al.* (2009) and Marongiu and Dimauro (2013), who reported that sexual behaviour was very poor in primiparous lactating rabbits.

Moreover, our results indicated that both bio-stimulation methods were positive and effective in all studied traits, but presence of a doe beside buck cage method was superior over the doe-litter separation method in most studied traits. Using the doe beside buck cage method had significantly higher frequency of male circling around female, female circling around male than those values obtained by doe-litter separation method. E_2 level and conception rate were significantly higher using this method than doe-litter separation method. This improvement may be due to higher serum concentrations of E_2 in female near buck cage method than in doe-litter separation method. McNitt (1992) and Berepubo *et al.* (1993) reported that presence of female beside buck is very important for the continuous contacts of visual, auditory, olfactory and tactile between them. This, in turn, activates the hypothalamus-pituitary-gonads axis to release GnRH and sexual hormones, which have a strong influence on rabbit sexual behaviour (Pau *et al.*, 1986; Bakker and Baum, 2000; Melo and Gonzalez-Mariscal (2010). On the other hand, prolactin hormone is increasing in suckling females compared to non-suckling females. McNitt (1992) reported that reduction of sexual behaviour during suckling period returned to the rise in prolactin, which has a negative effect on receptivity percentage. Moreover, highly positive correlations were found between E_2 concentration and sexual behavioural activities and physiological traits (Table 5). This may explain the lower response in most traits in suckling doe group (doe-litter separation treatment) compared to the non-suckling one (existence beside male cage treatment) in both breeds in our study.

Finally, our results clearly confirmed that significant differences, found in behavioural activities and physiological traits, are due to breeds of rabbits, irrespective of treatments. NZW does had significantly higher time of walking and lower time of standing behaviour than those recorded in BR does. Also, NZW breed had significantly increased frequency of actual mating, but insignificantly higher frequency of male circling around female, female circling around male and male mounting female, than those values recorded in the BR breed. Moreover, NZW does had insignificantly higher E_2 level and conception rate than BR does. Our results are in agreement with those obtained by Khalil *et al.* (2014), who reported that NZW breed had more active and higher reproductive efficiency than the BR breed, under the same managerial conditions. These differences between the two breeds may be due to genetic variation between exotic and local breeds.

CONCLUSION

Bio-stimulation methods resulted in positive changes in the basic and sexual behaviour, as well as improvement in the reproductive parameters of non-receptive female rabbits for mating. Therefore, these methods are strongly suggested to induce oestrus in rabbits instead of hormonal treatments. These methods are easy and cheap to apply, very consistent with animal welfare and well adapted to cyclic rabbit production systems at commercial intensive bases, as recommended by many rabbit European association for rabbit production and welfare.

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STABILIZING SELECTION FOR LOWER PHENOTYPE VARIABILITY OF RABBITS

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ABSTRACT

Stabilizing selection for lower variability of liveborn kits in a litter resulted in higher vitality and significantly ($P < 0.05$) higher mean number of weaned kits at 35 days of age. In the group of kits from selected mothers with higher variability per litter and coefficient of variation > 11 %, the mortality to weaning (35 days) and post-weaning (42 days) was 27.27 %. In the group from mothers with lower variability between litters and coefficient of variation < 11 %, the mortality within this period was zero. Females with lower variability of liveborn kits per litter and their kits at the 42nd day of age showed lower level of C-reactive protein (CRP) in blood plasma compared to the second group. Strong negative correlation (r = -0.795) was confirmed between the coefficient of variation of liveborn kits and the number of weaned kits with coefficient of determination $r^2 = 0.633$. In practice it means that higher balance or lower variability of the number of liveborn kits between the individual litters has positive influence on the number of weaned kits and this parameter was influenced on up to 63.3 %, while the rest of the influence (36.7 %) was entirely random. Strong negative correlation (r = -0.94) was noted also between the markers of milk yield in females up to the 21st day and the variation coefficient of liveborn kits. The determination coefficient in this case was $r^2 = 0.884$. These data suggest that higher stability of liveborn kits numbers between litters has a positive effect on the number of weaned kits of rabbits and also that targeted selection for lower levels of C-reactive protein in blood plasma can help to improve effective production through more effectively innate immunity against pathogens and therefore lower mortality of growing rabbits.

Key words: stabilizing selection; number of liveborn kits; milk yield; determination coefficient; vitality

INTRODUCTION

Selection at an early age for breeding and intensive production is an unavoidable prerequisite of a successful system for intensive animal production all around the world (Niranjan *et al*., 2010). Studies of multiple authors determined that direct as well as maternal influences are especially important for the intensity of growth of animals to weaning (Ferraz *et al*., 1992; Lukefahr *et al*., 1993; 1996) and affect the phenotype expression of young animals through the genotype of their mother for maternal behaviour and intensity of growth. On the other hand, in multiparity species such as rabbit, it was proven that the influence of the mother, which is expressed in the size of the litter and the birth weight, also influences the mortality in the period since milk nutrition to weaning of kits (Poigner *et al*., 2000). The presence of maternal antigens in the blood serum of rabbit kits is notable and detectable up to 6 – 9 weeks after birth, at a later age its levels are low with negligible effect (Blasco *et al*., 1983; Szendrö *et al*., 1984). Basing on the aforementioned, the number of weaned kits correlates with the genetic

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predisposition of the mother, her health status and the total breeding condition of the mother (Ondruska *et al*., 2016a; 2016b). The first factor usually affecting the impact of various diseases is the immunity of the given individual – host. One of the main characteristics of a health profile, immunologic and genetic predispositions is the C-reactive protein (CRP). The main characteristics of the biological action of CRP is its ability to bind phosphocholine, which allows the immune system to recognize foreign pathogens, as well as damaged or otherwise impacted cells of its own body. From the immunological standpoint, CRP acts as opsonin. CRP binds itself to the surface of the antigen and activates its phagocytose through immune cells. Opsonised can be, for example, bacteria or other pathogen organisms and afterwards, opsonins are recognized by Fc receptors, which are located on the surface of phagocytes (macrophages, neutrophiles) and they are phagocytosed and neutralised. CRP is, therefore, a ligand directly bound to neutrophiles, macrophages and other phagocyte cells, which stimulates inflammatory reaction and production of cytokines. The quick increase of CRP levels, as a result of induced stimulus, shows that CRP is a component of the inherent immunity response (Black *et al*., 2004).

Subject of the selection is always the individual's phenotype but not the genes. It is important to realize that selection for genes is always mediated by the phenotype, because biological fitness is a complex phenomenon. Fitness, as such, cannot be considered a trait; instead, it comprises a number of morphological and physiological traits, of which each can have its own hereditary basis. Selection, due to its impact on division of any trait affecting biological fitness, can therefore affect several genes at the same time. In stabilizing selection, the higher chance for survival and reproduction belongs to the individuals with values of the trait close to the population mean. Alleles that cause extreme values of the trait (high or low) are removed from the population, what leads to decreased variability from one generation to the next, while the population mean remains unchanged. For the evolutionary genetics, phenotypic variance is one of the selection key issue for several animal models.

Zhang and Hill (2005) found that the optimum phenotypes are selected for reproduction and less variable genotypes are favoured. Bodin *et al*. (2010) described the useful genetic uniformity in production traits in rabbits. They found correlations between the homogeneity of litter's birth weight and higher viability of the kits. This is a trait directly related to fitness; environmental variance can be related to the capacity of animals to cope with new environmental conditions (Blasco *et al*., 2017). Animals with less adaptable genotypes can be a more sensitive to diseases and stress and show a higher degree of variability in litter size (García *et al*., 2012; Argente *et al*. 2014; García *et al*. 2016).

For this reason, the main aim of the research programme was the application of standard selection procedures (1st stage of research) supplemented by results from immunologic testing by the ELISA method (2nd stage of research) in order to create suitable selection criteria to improve rabbits' vitality. The aim of the suggested selection procedures was to increase the vitality of young animals and the efficacy of rabbit production in extensive and intensive breeding systems.

MATERIAL AND METHODS

Experimental design, animals and management

All experiments were performed in accordance with relevant institutional and national guidelines for the care and use of animals, and all experimental procedures involving animals were approved by an ethical committee. The trial was performed at the accredited facility of experimental rabbit farm SK CH 17016 at the National Agricultural and Food Centre, Nitra, Slovak Republic.

The trials performed on clinically healthy rabbits were divided into two stages. In the first stage a total of 24 bucks (four different populations of rabbit meat lines; 6 bucks from each one) were involved. Totally, 164 pregnant does and 1513 kits from six different populations of rabbit meat lines were evaluated (Table 1). The production parameters (the number of liveborn kits, still-born kits and milk production until the 21st day of lactation) and the number of weaned kits at the age of 35 days were monitored.

For the milk yield determination, the indirect method of predicting milk production using formula based on a high correlation between milk production

and litter live weight gain at 21 days was used. Equation for the calculation of milk production of female rabbits is as follows:

Milk yield at 21 days (g) = 2 . (m21 – m0)
m21 – weight of the litter on the 21st day
m0 – weight of the litter after birth

During the second stage, stabilizing selection of females from the population with the best reproduction parameters from the first stage (P91 and M91) was performed in order to arrange two groups, which were then immunologically tested (ELISA) for CRP levels in blood plasma. The basic criterion to form the two groups of females from the original meat lines (M91 and P91) was the number of kits per litter in 3 – 9 litters, where females with 7 – 10 kits per litter were included into the CRP-1 group and females with higher variability of the number of kits per litter (1–15 kits) were in the group CRP-2. ELISA analyses were performed on blood plasma samples collected from 24 females of original meat lines of the rabbit bred. The females of the parental generation were divided into two groups: 1. Experimental group of females (12 animals) was subjected to strict stabilizing selection: minimum number of liveborn kits 7–10 per litter, in a minimum of three litters. 2. Control group (12 animals) had large variations in the number of liveborn kits per litter among at least three litters (1–15 kits per litter). The experimental females of all genotypes were at the age between 11 and 18 months. The does with kits were housed in cages made of spot-welded wire mesh of 560 × 760 mm in size (width × length) and with a resting area (560 × 310 mm) arranged in flat decks on one level. The nest (560 × 260 mm) was lined with sterile wood shavings. The nest area and cage were separated by a sheet metal wall with a door. The rabbits were fed a commercial diet (crude protein 177.25 $g.kg^{-1}$; crude fibre 168.28 $g.kg^{-1}$; fat 34.21 $g.kg^{-1}$; acid detergent fibre 185.21 $g.kg^{-1}$; neutral detergent fibre 316.19 $g.kg^{-1}$ and metabolizable energy 11.08 $MJ.kg^{-1}$). All animals had *ad libitum* access to feed. Drinking water was provided with nipple drinkers *ad libitum*. A cycle of 16 h of light and 8 h of dark was used throughout the trial. Temperature and humidity in the building were recorded continuously with a digital thermograph. Heating and forced ventilation systems allowed the building temperature to be maintained within 18 ± 4 °C throughout the trial. Relative humidity was in the interval of 70 ± 5 %.

Sample collection and ELISA analysis

Samples of peripheral blood for testing were taken from the *vena auricularis centralis* into heparinized tubes. Afterwards, 30 minutes after collection the samples were centrifuged at 1000 x g for 15 minutes at 4 °C to produce blood plasma for immunologic testing using ELISA (Enzyme Linked Immuno Sorbent Assay) for antigen detection. A commercial rabbit C-reactive ELISA kit (SunRed Bio, Shanghai, China; cat. No. 201-09-0003) with a detection range 50 $\mu g.L^{-1}$ – 1000 $\mu g.L^{-1}$ was used for CRP level determination. Values of CRP in rabbit blood plasma were measured using a PowerWave XS microplate spectrophotometer (Biotek) at the wave length of 450 nm.

Statistical analysis

The collected data were statistically analysed by a t-test using an Excel software and the commercial SAS 9.1 statistics package (SAS Institute Inc, USA). Statistical significance was indicated by *P*-values lower than 0.01 or 0.05. Correlations of production parameters were evaluated by Pearson Correlation Coefficient Calculator (https://www.socscistatistics.com/tests/pearson/Default2.aspx).

Coefficient of variance was calculated using the following formula:

$$v = \frac{sd}{\bar{x}} \cdot 100\ (\%)$$

v = coefficient of variance; sd = standard deviation; $\bar{x}$ = mean

RESULTS AND DISCUSSION

The results from the first stage of the experiment with long-term monitoring of production parameters (Table 1) showed strong negative correlation (r = -0.795) between the variation coefficients of liveborn kits and the number of weaned kits with the coefficient of determination $r^2 = 0.633$. In practice this means, that higher stability of litters or lower variability of liveborn kit numbers between litters has a positive effect on the number of weaned kits, and this parameter affects as much as 63.3 %

with the remaining influence (36.7 %) being entirely random. Strong negative correlation (r = -0.94) was determined also between the markers of milk yield of females before the 21st day and the coefficient of variation of liveborn kits. Coefficient of determination in this case was $r^2 = 0.884$.

Our results are in accordance with Blasco *et al*. (2017), who monitored litter size in two different rabbit lines: one line of rabbits for litter-size homogeneity and one line for litter-size heterogeneity by measuring intra-female phenotypic variance. Litter size was consistently larger in the low variance of litter size line than in the high variance of litter size line throughout the experiment. They found that the selection for reduced rabbit litter size variability does not decrease litter size.

In our study we determined strong positive correlation (r = 0.815) between the parameters of milk yield and the number of weaned kits with coefficient of determination $r^2 = 0.664$. These results are in line with several studies described the importance of milk as the essential and the only source of nutrition for kits in a first days after birth (Fortun-Lamothe and Gidenne 2000; Maertens *et al*. 2006). According to the study of Bonachera *et al*. (2017) the milk production in the first 21 days of lactation has a significant impact on the growth and health of the kits and is very important and the limiting factor for successful rearing during the pre-weaning period.

Table 1. Selected production parameters of the evaluated meat lines and breeds of rabbits

Parameter	Genotype (♂ x ♀)					
	Hy x B1	M91 x M91	P91 x P91	Hy x Hy	Ch x Ch	M91 x Bls
Number of kindled females	36	33	30	34	15	16
Total number of liveborn kits	360	304	247	330	128	144
Mean number of liveborn kits per litter	10.00 ± 1.96	9.21 ± 1.41	8.23 ± 1.38	9.71 ± 1.96	8.53 ± 1.96	9.00 ± 1.55
Mean number of weaned kits per litter	5.11 ± 1.78	6.3 ± 1.49	6.4 ± 1.58	4.79 ± 2.15	5.30 ± 1.77	6.20 ± 1.40
Coefficient of variation of the number of liveborn kits (%)	19.6	15.31	16.77	20.19	22.98	17.22
Milk yield (g)	4 190	5 030	5 170	4 340	3 810	4 660

Hy – Hycole rabbit hybrid; B1 – F2 generation of animals created during hybridization of standard local synthetic broiler line (M91) with sires of Belgian giant white; M91 and P91 – local synthetic broiler rabbit lines; Ch – Chinchilla giganta; Bls – Big light silver.

During the second stage, using stabilizing selection two groups were formed from the population's females (P91 and M91) with the best reproduction parameters from the 1st stage. They were then immunologically tested (ELISA) for CRP levels in blood plasma. Stabilizing selection of females was performed in relation to variability of the number of liveborn kits. Additionally, we also evaluated other selected reproduction parameters (conception rate, mean number of liveborn kits, vitality of kits after weaning (42 days of age) and the levels of CRP in blood plasma of females.

C-reactive protein (CRP), as one of the main components of the inherited immunity mechanism, is in the hierarchy of proteins the first to be produced in the liver during the acute phase of the immune response or in the case of any invasion by a pathogen or immunostimulation (Figure 1). Targeted selection for lower levels of C-reactive protein (CRP) in blood plasma will help to improve effective production of rabbits due to innate immunity against pathogens and, therefore, lower levels of mortality especially after weaning (Table 2).

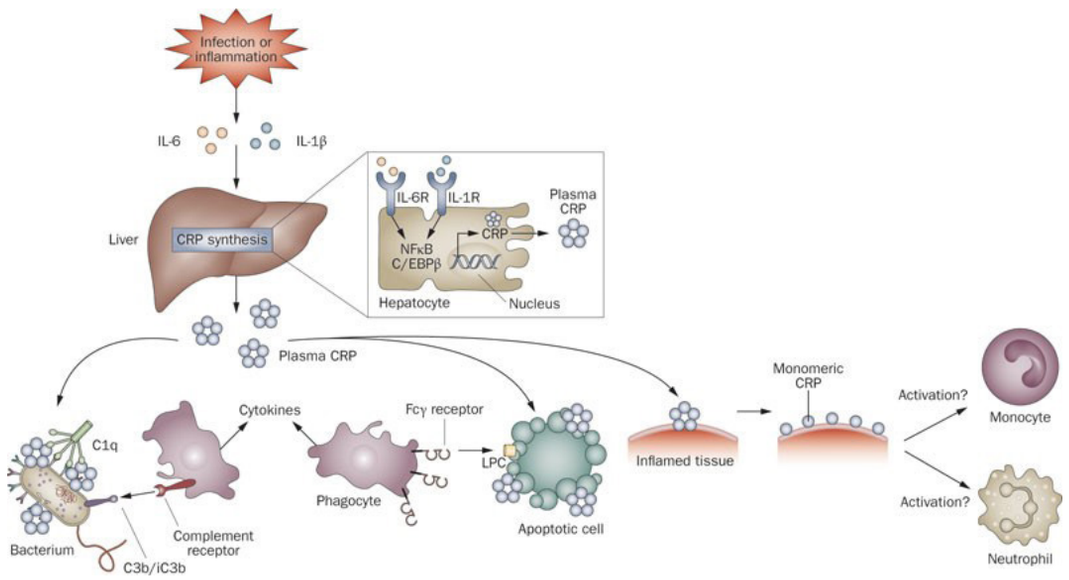

Figure 1. Synthesis and expression of C-reactive protein

From the results presented in Table 2 it is evident that stabilizing selection for lower variability of liveborn kits per litter led to higher vitality and significantly ($P < 0.05$) higher mean numbers of kits weaned at the age of 35 days. In the group of kits from selected mothers with higher variability per litter, with the coefficient of variation > 11 %, the mortality to weaning (35 days) and the post-weaning period (42 days) was 27.27 %, while in the group from mothers with lower variability per litter with coefficient of variation < 11 % the mortality over the same period was zero. Females with lower variability of liveborn kits per litter and their kits on the 42nd day showed lower level of C-reactive protein (CRP) in the blood plasma compared to the other group. These results are in line with Blasco *et al*. (2017), who found higher and more effectively tolerated of line with litter size homogeneity for external stressors than the line selected for litter size heterogeneity. The line with high variance of litter size and a higher subclinical immune response, according to Rauw (2012), is related to a greater sensitivity to diseases or to less tolerance to common microorganisms in the farm microenvironment. On the other hand, García *et al*. (2012) and Argente *et al*. (2014) recorded the faster and stronger response to invading agents in rabbit line with low variance of litter size.

Table 2. Selected production parameters of meat rabbit populations after stabilizing selection

Group	Conception rate (%)	Total number of liveborn kits	Mean number of liveborn kits per litter $\bar{x} \pm sd$	Mean number of weaned kits per litter $\bar{x} \pm sd$	CRP levels in mothers' blood plasma (μg.L^{-1}) $\bar{x} \pm sd$	Coefficient of variation of liveborn kits (%)	CRP levels in plasma of growing rabbits (μg.L^{-1}) $\bar{x} \pm sd$
CRP–1	84.61	78	7.8 ± 0.84	7.8 ± 0.84	129.45 ± 24.75	10.77	56.78 ± 7.6
CRP–2	81.81	67	6.60 ± 3.05	4.80 ± 3.27	143.58 ± 50.82	46.21	120.69 ± 46.93
t-test	-	-	-	*	-		***

*Difference is statistically significant at $P < 0.05$; ***Difference is statistically significant at $P < 0.001$.

CONCLUSION

The obtained results objectively verify the hypothesis of genetic and immunologic research about the application of stabilizing selection of rabbits at the level of variability of the liveborn kit number and concentrations of C-reactive protein in the blood plasma of rabbits, as well as their direct relationship to better vitality of kits before weaning and the reproduction fitness of the selected females (high milk production up to the 21st day). At the same time, positive impact of the given breeding programme, involved in the selection program, on the economy and efficiency is clearly declared, as with the same costs and inputs a higher number of weaned animals is achieved. Based on these results, we recommend breeding of meat line rabbits under strict stabilizing selection of females of the founding herd after a minimum of 2 litters for low variability of liveborn kits (7–10 animals) in the litter with a coefficient of variation less than 11 %.

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DEPLETION OF DEAD SPERMATOZOA DID NOT SUFFICIENTLY IMPROVE THE QUALITY OF RAM SEMEN: SHORT COMMUNICATION

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ABSTRACT

The main objective of this study was to improve the motility and overall quality of spermatozoa from the fresh ram semen samples using more sensitive depletion programmes of AutoMACS Pro Separator to remove dead sperm cells. Briefly, ram spermatozoa, given either at high (10^8) or low (10^7) concentrations, were incubated with the Dead Cell Removal kit reagent and magnetically sorted using two very sensitive depletion programmes (Depl05 and Depl025) with different sample loading rates. Fresh unsorted semen samples (control) as well as both sorted fractions (negative and positive) were analysed using computer-assisted sperm assay (CASA) to assess the motility parameters and using flow cytometry to evaluate the proportion of dead cells and overall magnetic-activated cell sorting (MACS) efficiency. We obtained significantly ($P < 0.01$) lower percentage of dead spermatozoa only after sorting the high concentrated spermatozoa using a Depl025 programme compared to control samples. However, the negative fractions still contained more than 20 % of dead cells irrespective of the sorting programme used. In addition, the motility parameters were significantly improved neither by the used sorting strategy nor by the adjustment of spermatozoa concentration. In conclusion, further optimization of this method is required in order to sufficiently remove dead spermatozoa and to improve the spermatozoa motility.

Key words: ram semen; CASA; MACS; depletion; dead spermatozoa; flow cytometry

INTRODUCTION

In general, assisted reproductive technology (ART) is commonly used to solve the infertility in humans (Tournaye, 2012) or on commercial animal breeding farms (Niemann *et al*., 2011). One of the ART techniques is also a cryopreservation of spermatozoa. However, it has been reported recently that increased presence of dead spermatozoa in raw semen is associated with poor cryopreservation and insufficient outcomes of *in vitro* fertilization (IVF) (Roca *et al*., 2013) compared with the spermatozoa from a standard semen sample (Oehninger *et al*., 2000; Di Santo *et al*., 2011). On the other hand, sub-standard semen samples are usually characterized by the presence of a large population of morphologically altered and/or dead spermatozoa (Esteves *et al*., 2012).

Magnetic-activated cell sorting (MACS) also belongs to ART techniques that has been used in human assisted reproduction (Said *et al*., 2006; Oseguera-López *et al*., 2019) to eliminate spermatozoa with deteriorated membranes and apoptotic-like features (Grunewald *et al*., 2001) mainly in humans (Glander *et al*., 2002; Agarwal *et al*., 2009; Vendrell *et al*., 2014; Bucar *et al*., 2015) but also in animals (Vasicek *et al*., 2014a; b; Mrkun

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et al., 2014) using manual MASC instruments. We have already applied this technique for the first time for ram semen samples that were sorted using fully automated magnetic sorter (Vašíček *et al*., 2020). However, the used depletion programmes (Deplete and Depletes) were not sensitive enough to efficiently remove dead spermatozoa from the fresh semen samples. Moreover, the spermatozoa motility, which is one of the primary indicators of semen quality (Baláži *et al*., 2020), was not improved after sorting. Therefore, in this study in order to improve sperm motility we used more sensitive depletion programmes for the elimination of dead spermatozoa.

MATERIAL AND METHODS

Clinically health and sexually mature rams of Native Wallachian (n = 2) and Improved Wallachian (n = 1) sheep breeds aged 2 – 4 years were used in this study. Rams were kept under external conditions in individual stalls at the breeding facility (NPPC, RIAP Nitra, Lužianky, Slovak Republic). They were fed with hay bale and oats; water and mineral salt were supplied *ad libitum*. Prior to the experiment, semen samples were collected by an electro-ejaculation once a week for the duration of several weeks, as described previously (Baláži *et al*., 2020). All semen collections were realized in autumn. Ram semen samples immediately after collection were transferred to the laboratory in a water bath for the subsequent processing. The experiments were carried out in accordance with the Code of Ethics of the EU Directive 2010/63/EU for animal experiments.

Freshly collected semen samples were diluted and analysed by CASA (Sperm Vision™, MiniTübe, Germany) for concentration (10^9 per mL), total motility (motility > 5 µm.s^{-1}) and progressive motility (motility > 20 µm.s^{-1}) of spermatozoa, as described previously (Baláži *et al*., 2020). The CASA analyses were performed again after MACS sorting only in negative fractions.

In this study, we performed four types of experiments in order to determine the most appropriate selection strategy and the number of sorted spermatozoa. At first, ram spermatozoa at high concentration (10^8) were sorted using two different sensitive depletion programmes (Depl05 and Depl025; first and second type of experiment, respectively). Next, ram spermatozoa at low concentration (10^7) were sorted using aforementioned programmes (third and fourth type of experiment, respectively). Briefly, aliquots of each semen samples (high and low concentrated) were diluted in 1 or 0.5 ml of a reagent from the Dead Cell Removal kit (Miltenyi Biotec, Germany), respectively and incubated for 15 min at room temperature according to the producer's manual. After incubation, dead spermatozoa were removed from the ram semen samples by AutoMACS Pro Separator (Miltenyi Biotec, Germany). A HEPES buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, at pH 7.2) was used as a sheath fluid instead of the standard MACS running buffer, since the commercial kit required a buffer with calcium for a proper binding of nanoparticles to the cells. The both depletion programmes (Depl05 and Depl025) differ mainly in the loading rate. The Depl05 programme loads 0.5 mL of sample per minute, while the Depl025 provides slower loading rate (0.25 mL.min^{-1}).

The negative and positive fractions obtained from MACS sorting were stained with allophycocyanin (APC)-conjugated labelling check reagent (LCR; Miltenyi Biotec, Germany) in order to determine the proportion of spermatozoa with bound nanoparticles. Moreover, all sorted samples, including fresh unsorted (control) samples, were mixed with a propidium iodide (PI at 50 µg.mL^{-1}; Molecular Probes, USA) prior to the analysis in order to reveal the dead spermatozoa. Stained samples (at least 10,000 cells) were immediately analysed using a FACSCalibur flow cytometer (BD, San Jose, CA, USA).

The experiments were replicated for three times. Semen samples, which contained more than 50 % of dead spermatozoa, were used for the immunomagnetic sorting. Motility parameters and the percentage of dead spermatozoa were statistically evaluated using a GraphPad Prism version 9.0.2 for Windows (GraphPad Software, San Diego, CA, USA) with a one-way ANOVA (Dunnett's and Sidak's method, resp.) and expressed as the mean ± SEM. P-values at $P < 0.05$ were considered as statistically significant.

RESULTS AND DISCUSSION

We tested the possibility of the potential use of immunomagnetic dead sperm removal to improve the semen quality. Two different depletion strategies (Depl05 and Depl025) for very sensitive fully automated cell sorting were examined. Ram spermatozoa were sorted at two different concentrations (10^8 and 10^7) in order to define a proper concentration according to the column capacity. High concentrated samples (10^8) showed significantly reduced dead cell number in negative fractions in comparison to control, when separated using Depl025 programme ($P < 0.01$). On the other hand, the proportion of dead spermatozoa also decreased slightly but insignificantly after sorting using a Depl05 programme. However, negative fractions of those samples still contained more than 20 % of dead spermatozoa. On the other hand, the proportion of dead spermatozoa in positive fractions using both programmes, Depl05 and Depl025, increased significantly (over 80 %; $P < 0.05$ and $P < 0.001$, respectively) in comparison to the control samples (Figure 1A). On the contrary, no significant changes in the proportion of dead cells were observed either in negative or positive fractions of low concentrated semen samples (10^7 of cells) irrespective of a used sorting programme (Figure 1B). Therefore, decreased concentration of spermatozoa did not facilitate their sorting, and ram spermatozoa could be effectively sorted at the higher concentration (10^8 of cells).

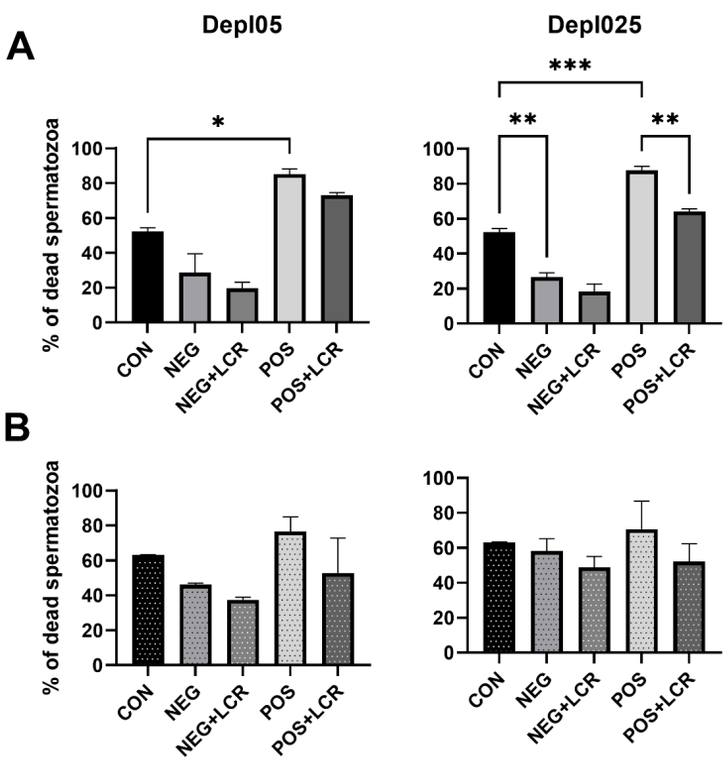

Figure 1. Changes in the proportion of dead spermatozoa after MACS sorting of high concentrated (A) and low concentrated (B) ram semen samples using two depletion programmes

Depl05 – sorting programme with loading rate at 0.5 mL.min^{-1}; Depl025 – sorting programme with loading rate at 0.25 mL.min^{-1}. Percentage of dead cells was determined as a proportion of the spermatozoa positively stained with a propidium iodide; CON – fresh semen samples before sorting; NEG – negatively sorted spermatozoa; NEG+LCR – negatively sorted spermatozoa co-stained with a labelling check reagent (LCR); POS – positively sorted spermatozoa; POS+LCR – positively sorted spermatozoa co-stained with a labelling check reagent (LCR); * – statistical significance at $P < 0.05$; ** – statistical significance at $P < 0.01$; *** – statistical significance at $P < 0.001$.

Interestingly, a slightly lower proportion of spermatozoa positive for both, PI and LCR, was observed in all samples (NEG+LCR and POS+LCR), when compared to the total percentage of dead (PI positive) cells in the NEG and POS fractions (Figure 1). Similar difference, though significant ($P < 0.01$), was noticed between positive fractions in high concentrated samples sorted using Depl025 (Figure 1A). This might indicate, that some portion of dead cells was not detected by the nanoparticles, and/or that those dead cells were appeared as a result of the sorting procedure itself, since ram spermatozoa are very sensitive to any physiological changes.

The aim of this study was to remove the dead cells from the semen samples in order to improve their quality in terms of better motility parameters. However, the total and progressive motility of high concentrated spermatozoa slightly decreased after sorting by a Depl05 programme. On the other hand, the progressive motility of spermatozoa slightly but insignificantly increased after sorting using a Depl025 programme (Figure 2A). Furthermore, both motility parameters in low concentrated semen samples had decreasing tendency after sorting using both programmes (Figure 2B). The same observation was reported in our previous study (Vašíček *et al*., 2020), where the motility of ram spermatozoa after depleting the dead cells using fewer sensitive programmes (Deplete and Depletes) decreased insignificantly, and the proportion of dead cells in negative fractions was not changed, when compared to the control samples. Similarly, another our study (Vasicek *et al*., 2014b), focused on the removal of dead cells from rabbit semen samples using the manual MACS instruments, did not result in the improving the motility in negative fractions, as well as did not significantly decrease the number of dead spermatozoa.

On the contrary, in this study we used fully automated sorter in order to facilitate the sorting

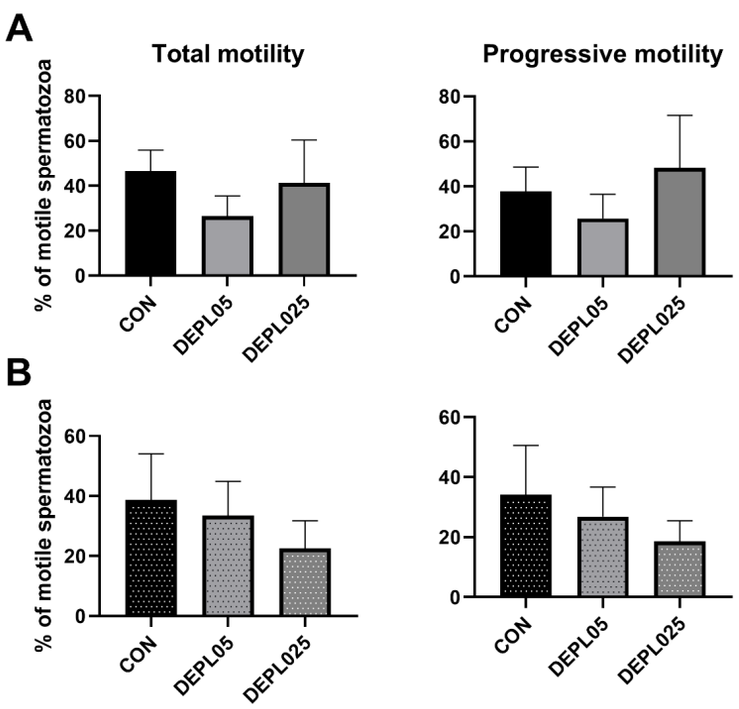

Figure 2. Changes in the motility parameters of spermatozoa after MACS sorting of high concentrated (A) and low concentrated (B) ram semen samples using two depletion programmes

CON – fresh semen samples before sorting; Depl05 – negative fractions of spermatozoa sorted using programme with loading rate at 0.5 mL.min^{-1}; Depl025 – negative fractions of spermatozoa sorted using sorting programme with loading rate at 0.25 mL.min^{-1}.

process and make it more sensitive to ram spermatozoa. Nevertheless, manual MACS instruments are commonly used to remove dead or apoptotic spermatozoa from human semen samples by this technique with variable results (Paasch *et al*., 2003; Delbès *et al*., 2013; Grunewald and Paasch, 2013; Merino-Ruiz *et al*., 2019). Paasch *et al*. (2003) demonstrated an insignificant loss of progressive motility in negative fractions after MACS. On the other hand, a significant improvement in the quality (including motility) of negatively sorted spermatozoa in combination with density gradient centrifugation was observed by Delbès *et al*. (2013) and Merino-Ruiz *et al*. (2019).

Although we obtained a significant reduction in the number of dead spermatozoa in high concentrated ram semen samples after MACS (Depl025), nevertheless their presence in samples was still more than 20 %. It has been reported that high proportion of dead spermatozoa in fresh semen samples significantly increased ROS generation and nuclear DNA fragmentation in frozen-thawed boar spermatozoa and, thus, negatively affected the IVF outcomes (Roca *et al*., 2013). Therefore, it is important to find an optimal method to decrease dead spermatozoa number in fresh ram semen of valuable breeding males to the minimum.

CONCLUSION

Present study indicates that fresh ram spermatozoa with high proportion of dead cells (over 50 %) can be free of these cells using a very sensitive depletion programme (Depl025), when MACS sorted at high concentrations. However, a considerable proportion of dead cells (over 20 %) still remains in semen samples after sorting. Therefore, further studies are needed in order to optimize this method and significantly improve the quality of ram semen samples, what could increase the cryosurvival rates and IVF outcomes of frozen-thawed spermatozoa.

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